RESEARCH ARTICLE

Immunotherapeutic Potential of Eugenol Emulsion in Experimental Visceral Leishmaniasis

Mohammad Islamuddin1a, Garima Chouhan1b, Muzamil Yaqub Want1c, Hani A. Ozbak2, Hassan A. Hemeg2, Farhat Afrin2*

1 Parasite Immunology Laboratory, Department of Biotechnology, Jamia Hamdard (Hamdard University), New Delhi, India, 2 Department of Medical Laboratories Technology, Faculty of Applied Medical Sciences, Taibah University, Medina, Kingdom of Saudi Arabia

a Current address: Molecular Virology and Vaccinology Laboratory, Department of Biotechnology, Jamia Hamdard (Hamdard University), New Delhi, India;
b Current address: Department of Biotechnology, School of Engineering and Technology, Sharda University, Greater Noida, Uttar Pradesh, India.
c Current Address: Immuno-Biology Laboratory, Centre for Human Microbial Ecology, Translational Health Science and Technology Institute, NCR Biotech Science Cluster, Faridabad, Haryana, India

* farhatafrin@gmail.com, faislam@taibahu.edu.sa

Abstract

Background

The therapy of visceral leishmaniasis (VL) is limited by resistance, toxicity and decreased bioavailability of the existing drugs coupled with dramatic increase in HIV-co-infection, non-availability of vaccines and down regulation of cell-mediated immunity (CMI). Thus, we envisaged combating the problem with plant-derived antileishmanial drug that could concomitantly mitigate the immune suppression of the infected hosts. Several plant-derived compounds have been found to exert leishmanicidal activity via immunomodulation. In this direction, we investigated the antileishmanial activity of eugenol emulsion (EE), supplemented with its immunomodulatory and therapeutic efficacy in murine model of VL.

Methodology/Principal Findings

Oil-in-water emulsion of eugenol (EE) was prepared and size measured by dynamic light scattering (DLS). EE exhibited significant leishmanicidal activity with 50% inhibitory concentration of 8.43±0.96 μg ml⁻¹ and 5.05±1.72 μg ml⁻¹, respectively against the promastigotes and intracellular amastigotes of Leishmania donovani. For in vivo effectiveness, EE was administered intraperitoneally (25, 50 and 75 mg/kg b.w./day for 10 days) to 8 week-infected BALB/c mice. The cytotoxicity of EE was assessed in RAW 264.7 macrophages as well as in naive mice. EE induced a significant drop in hepatic and splenic parasite burdens as well as diminution in spleen and liver weights 10 days post-treatment, with augmentation of 24h-delayed type hypersensitivity (DTH) response and high IgG2a:IgG1, mirroring induction of CMI. Enhanced IFN-γ and IL-2 levels, with fall in disease-associated Th2 cytokines (IL-4 and IL-10) detected by flow cytometric bead-based array, substantiated the Th1...
immune signature. Lymphoproliferation and nitric oxide release were significantly elevated upon antigen revoke in vitro. The immune-stimulatory activity of EE was further corroborated by expansion of IFN-γ producing CD4+ and CD8+ splenic T lymphocytes and up-regulation of CD80 and CD86 on peritoneal macrophages. EE treated groups exhibited induction of CD8+ central memory T cells as evidenced from CD62L and CD44 expression. No biochemical alterations in hepatic and renal enzymes were observed.

Conclusions
Our results demonstrate antileishmanial activity of EE, potentiated by Th1 immunostimulation without adverse side effects. The Th1 immune polarizing effect may help to alleviate the depressed CMI and hence complement the leishmanicidal activity.

Author Summary
In the absence of vaccines and escalating resistance to the available chemotherapeutic drugs, alternate therapeutic options are urgently needed for visceral leishmaniasis (VL) or kala-azar, a systemic and potentially fatal, vector-borne neglected tropical disease, caused by Leishmania donovani. In the present study, we explored the two-prong effect of eugenol emulsion (EE) in eliminating the parasites with synergistic boosting of the dampened immune system, characteristic of active disease. Infected BALB/c mice upon intraperitoneal administration of EE, exhibited a significant decline in liver and spleen parasite load with concomitant drop in splenomegaly. Protection in treated mice coincided with Th1 immunopotentiation as was evident from substantial DTH and lymphoproliferative responses, elevated levels of IgG2a over IgG1 isotypes, significant enhancement in IFN-γ producing CD4+ and CD8+ T cells, waning levels of IL-4 and IL-10, augmented nitrite levels, induction of immunological memory and stimulation of antigen presenting capacity of macrophages, compared to infected mice. The dual antileishmanial and immunostimulatory potential of EE with no adverse toxic effects validates it as an adjunct to chemotherapy that may aid in leishmanicidal activity via ameliorating the depressed cellular immunity.

Introduction
Leishmaniasis, a complex vector-borne parasitic syndrome, is caused by obligate intra-macrophagic protozoan parasites of the genus Leishmania, a member of the order Kinetoplastida. The array of manifestations varies from a self-limiting cutaneous form to a potentially lethal visceralizing infestation of the liver, spleen and bone marrow [1]. Resolution of disease correlates with Leishmania-specific CD4+ type 1 T helper (Th1) and CD8+ T lymphocyte responses with production of interferon-γ (IFN-γ), macrophage nitric oxide (NO) and reactive oxygen species (ROS) generation [2–3]. Leishmania species exploit discrete mechanisms to elude the cellular immune defenses, such as inhibition of phagolysosomal fusion, and reactive nitrogen species (RNS)- and ROS-mediated macrophage microbicidal effects, dampening of cell-mediated immune response via blockade of antigenic peptide display to T cells, impaired secretion of Th1 cytokines, and infiltration of IL-10 producing T regulatory cells [4–6]. The treatment of visceral leishmaniasis (VL) is complicated because of intra-macrophagic refuge of the amastigotes, rendering the patient immunodeficient and unable to eliminate the parasites through the
natural defense mechanisms [7]. The quandary of VL has been compounded due to concomitant infection in acquired immunodeficiency syndrome (AIDS) patients [8].

There is no vaccine available against VL though several are in phase III clinical trials [9]. The existing anti-leishmanial therapy suffers from grave impediments such as drug resistance, compromising efficacy, toxicity, prolonged courses and parenteral routes of administration [10–12]. Hence new drugs for the treatment of VL are imperative. In an ongoing quest for safe and cheap antileishmanial agents, plant-based secondary metabolites are gaining ground [13–15]. The use of plant products as immune-stimulants has a traditional history. Treatment of leishmaniasis with natural or synthetic molecules appears to be dependent upon the development of an effective immune response that activates macrophages and lymphocytes to release their effector molecules [16–17].

A plethora of studies have reported immunomodulation with plant secondary metabolites such as licarin A, [18], niranthrin [19], alkaloid skimmianine [20], quassin [21], tannins and structurally related compounds [22], N-Palmitoyl-S-(2,3-bis(palmitoyloxy)-(2RS)-propyl)-Cys-Ser-Lys4 hydrochloride (Pam3Cys) [23] and linalool component of essential oil [24]. Synergistic antileishmanial and immunopotentiating effects of plant fractions or compounds have also been documented [25]. This may result in enhanced clearance of the parasites coupled with boosting of the depressed immunity associated with active VL.

Eugenol (Fig 1) is the major constituent of *Syzygium aromaticum*. *S. aromaticum* or common clove, is indigenous to tropical America and Australia [26] and is endowed with antibacterial [27] and anti-trypanosomal activities [28]. The extracts from flower buds of *S. aromaticum* has been reported to display antimalarial efficacy [29]. The immunomodulatory effect of *S. aromaticum* essential oil has been attributed in augmentation of humoral and cell mediated immune responses [30]. We have previously evaluated the leishmanicidal effect of eugenol-rich essential oil of *S. aromaticum* against promastigotes and intramacrophagic-amastigotes of *L. donovani* [31]. However, the poor solubility and high volatility limits its stability resulting in paradigm shift from therapeutic use of most oils to biocompatible emulsifiers. The antibacterial and antifungal activities of eugenol emulsions have been explored [32–33]. Encouraged by the above studies, we evaluated the antileishmanial and immunomodulatory potential of eugenol emulsion (EE) against experimental VL in BALB/c mice.

**Materials and Methods**

Eugenol, RPMI 1640 medium, M-199 medium, penicillin G sodium salt, streptomycin sulphate, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), carboxyfluoresceinsuccinimidyl ester (CFSE), AmB, anti-mouse IgG and isotype antibodies, o-phenylenediaminedihydrochloride (OPD) were procured from Sigma-Aldrich (St Louis, MO, USA). Fetal bovine serum
(FBS) was obtained from Gibco-BRL, DMSO from SRL, methanol from Merck, limulus amebocyte lysate (LAL) kit from Pierce, Thermo Scientific. Fluorochrome conjugated anti-mouse antibodies such as CD4-phycoerythin (PE), CD8-fluorescein isothiocyanate (FITC), CD80-allophycocyanin (APC), CD86-phycocerythrin cyanine dye 7, APC-CD4 and PE-CD8, FITC-IFN-γ, CD8-APC, CD62L-PE and CD44-FITC, isotype controls and Brefeldin A and cytokine bead array kit (CBA) were procured from BD Pharrmingen, USA. Aspartate aminotransaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), creatinine and urea kits were purchased from Span Diagnostics Ltd (Surat, Gujarat, India). Besides these, the analytical grade reagents were used.

Ethics Statement

The experiments were performed on female BALB/c mice, aged about 6–8 weeks (20–25 g) while the *L. donovani* parasites were maintained in Syrian golden hamsters (4–6 weeks old), after prior assessment and approval of the study protocol (Ethics Clearance number 459) by the Jamia Hamdard Animal Ethics Committee (JHAEC). JHAEC is registered under the Committee for the purpose of control and supervision of experiments on animals (CPCSEA) that is registered under Animal Welfare Division of the Central Government of India. The animals were kept in the Central Animal House facility of Hamdard University as per the CPCSEA guidelines. The animals were housed in standard size polycarbonate cages in groups of at least 5 mice (or 3 hamsters) per cage, with temperature maintained at 23 ± 1°C, relative humidity (55 ± 10%), 12:12 h light: dark cycle with *ad libitum* access to a standard pellet diet (Ashirwad Industries, Chandigarh, India) and drinking water. For *ex vivo* experiments, RAW 264.7 cells were procured from National Centre for Cell Science, Pune, India. Undesirable side effects of EE for painful abdominal distention and the resulting distress have been avoided by daily monitoring. We have used 0.15 ml for the intraperitoneal injection of EE in mice, that is, below the recommended volume (0.2 ml) for intraperitoneal administration of emulsion in mice.

*Leishmania* culture and maintenance

*Leishmania donovani* (MHOM/IN/AG/83) promastigotes were cultured in M199 medium, supplemented with 10% heat-inactivated FBS, 2 mM glutamine, 100 units/ml penicillin G sodium, and 100 μg/ml streptomycin sulphate at 22°C. The parasites were maintained in culture for 4–5 days (initial inocula being 1X10⁶ parasites/ml) and the late log or stationary phase promastigotes were harvested after the second or third passage.

Cell culture

Murine macrophage-like cells, RAW 264.7 were maintained at 37°C in 10% FBS-supplemented RPMI-1640 medium (pH 7.4) for 48–72 h in a humidified atmosphere with 5% CO₂. The subconfluent cultures (70–80%) were split in fresh medium split at 2 x 10⁵ cells ml⁻¹.

Formulation and characterization of Eugenol emulsion

Eugenol was procured from Sigma-Aldrich and oil-in-water emulsion (EE) was prepared using non-ionic surfactant, Tween 80 (T-80) and water to achieve a final concentration of oil mixture in the emulsion as 200 mg ml⁻¹. Emulsion was formulated by mixing Eugenol and surfactant, 4% micellar solution was prepared with T-80 and deionized water. Eugenol was poured into the micellar solution through calibrated micropipette under continuous stirring. The formulated emulsion was analyzed for particle size by dynamic light scattering (DLS) Nano-S90
Nanoseries, Malvern Instruments, UK). The samples were measured at 25°C with a fixed angle of 90°. The dosing emulsion was carefully stored in aluminium foil covered-glass vials at 4°C prior to biological assays.

**Dose-dependent anti-promastigote activity for determination of IC$_{50}$**

Promastigotes (2 x 10$^6$ cells ml$^{-1}$) were incubated for 72 h at 22°C without or with serial three-fold dilutions of EE, starting from 100 μg ml$^{-1}$ (100, 33.33, 11.11, 3.70, 1.23 and 0.41 μg ml$^{-1}$). The mean percent (%) viability was calculated by MTT assay. The inhibitory concentration responsible for 50% reduction in promastigote growth (IC$_{50}$), was graphically extrapolated by plotting percent (%) viability versus drug concentration. Surfactant, T-80 (0.00175%, present in 100 μg ml$^{-1}$ EE) was used as negative control.

**Ex vivo anti-amastigote activity and NO release from macrophages**

To assess the effects of EE on intracellular *L. donovani* amastigotes, RAW 264.7 macrophages (1x10$^6$ cells ml$^{-1}$) were infected with parasites in late log or stationary phase (cell/promastigote ratio, 1/10) at 37°C for 24 h. Thereafter, the non-ingested parasites were gently aspirated, and the infected macrophages were further incubated with EE (0–100 μg ml$^{-1}$) and with negative control (surfactant) for 48 h. The cells were then fixed, Giemsa-stained and microscopically evaluated for percent amastigote infectivity. At least 200 macrophages were enumerated per coverslip, and the concentration of EE that reduced amastigote infectivity by 50% (IC$_{50}$) was calculated.

In parallel, the nitrite concentration in the cell culture supernatants was analysed using Griess reaction. Briefly, to the harvested supernatant, an equal volume of Griess reagent (0.1% N-1-naphthyl ethylenediaminedihydrochloride and 1% sulphanilamide in 5% phosphoric acid) was added. After incubation for 10 min at room temperature (RT), the absorbance values were read at 550 nm, and nitrite concentration was calculated by performing linear regression of a standard curve of sodium nitrite. EE and all the reagents were analyzed for endotoxin, lipopolysaccharide (LPS) by chromogenic Limulus Amoebocyte Lysate (LAL) kit according to the manufacturer's instructions (Pierce, Thermo Scientific) and were found to be free of LPS (0.2 ng ml$^{-1}$ endotoxin).

**MTT cytotoxicity assay ex vivo**

To determine the adverse cytotoxic effects of EE, RAW 264.7 cells in RPMI 1640 medium were incubated for 48 h at 37°C in a humidified 5% CO$_2$ incubator with increasing concentrations of EE (0–200 μg ml$^{-1}$). Surfactant was used as negative control. MTT assay was used to evaluate cell viability, expressed as a percentage relative to untreated macrophages as control.

**In vivo antileishmanial potential of EE in *L. donovani* infected BALB/c mice**

Six to eight weeks old BALB/c mice were injected with 2x10$^7$ stationary phase *L. donovani* promastigotes in the lateral tail vein. At eight weeks of infection, after confirming the parasite load in three randomly selected animals; the mice were arbitrarily divided into four groups of 10 animals each (A- D). Group A comprised of untreated infected mice (INF); Group B-Saline administered vehicle control mice (VC, i.p). Groups C, D, E (EE, i.p.), Infected mice that received respectively three doses of EE (25/50/75 mg/kg body weight) each day for 10 days. Group F- treated with Amphotericin B (AMB, 5 mg/kg b.w., i.v., alternately over a ten day period), worked as the positive control. Ten days post-treatment, mice were euthanized by...
carbon dioxide asphyxiation for enumeration of hepatic and splenic amastigote burden that was expressed in terms of Leishman Donovan Units (LDU). LDU was evaluated per organ from giemsa-stained multiple impressions smears [23] as the number of amastigotes per 500 host cells X organ weight (mg). Percent reduction in amastigote load (% protection) was calculated as the difference between LDU of infected control and treated mice/ LDU of infected control X 100. Protection coincided with a drop in hepatosplenomegaly and parasite clearance with respect to untreated infected controls [37].

Freeze-thawed (FT) and soluble leishmanial antigen (SLA) preparation

SLA and FT antigens were prepared from promastigotes in the stationary-phase [38–39]. Briefly, promastigotes in third or fourth passage were harvested, washed four times in cold 1X PBS and resuspended at 2x10^8 cells ml^-1. The suspension was frozen at -80°C (30 min) and thawed in a 37°C water bath (15 min) alternately for 6 cycles. For SLA preparation, after ten alternate freeze-thawing cycles, the suspension was centrifuged (5250 x g, 4°C, 10 min) and the supernatant containing leishmanial antigens (SLA) harvested. FT and SLA were stored at -70°C until use and the protein quantitated [40].

Induction of delayed-type hypersensitivity (DTH)

DTH response in infected mice subsequent to treatment was evaluated as a hallmark of cellular immunity. Briefly, two days prior to euthanisation, mice were intradermally injected with FT (50 μl: 800 μg ml^-1) in the right footpad and PBS in the left footpad. After 48 h, the thickness of footpads was recorded using vernier calipers and the results expressed as the difference in swelling of the right compared to the contralateral left hind footpad [39, 41].

Determination of serum IgG1 and IgG2a isotypes

The Leishmania-specific serum IgG subclasses were measured through enzyme-linked immunosorbent assay (ELISA). In brief, FT (0.25 μg/well) was seeded in the wells of ELISA plates (Nunc, Roskilde, Denmark) for 1 h at 37°C. After three washes, blocking was done with 1% BSA for 2 h at RT followed by addition of 1,000-fold diluted mice sera. Post-washing, IgG1 and IgG2a isotype-specific goat anti-mouse secondary antibodies (Sigma Aldrich) were added and the plates incubated at 37°C for 1 h. After washing, incubation with peroxidase-conjugated rabbit anti-goat IgG as the tertiary antibody (Sigma Aldrich) was done at 37°C for 1 h. Post-washing, OPD was added and the absorbance taken on an ELISA plate reader at 490 nm [41].

Nitrite estimation

Culture supernatants of peritoneal macrophages from normal and infected mice following treatment were analyzed for LPS- and SLA-specific nitrite (NO2^-) levels by the Griess method as described previously [42]. Briefly, to the culture supernatants, equal volume of Griess reagent was added and incubated for 15 min at RT. The optical density (OD) was measured at 550 nm using a microplate reader. Dilute solution of sodium nitrite (NaNO2) in culture medium served as a standard. All the reagents and fractions were free of LPS (0.2 ng/ml endotoxin) as confirmed by LAL assay.

Lymphocyte proliferation

To assess the effect of EE on the proliferation of lymphocytes, single cell suspension from spleens (5 × 10^6 cells ml^-1) and lymph nodes (2 × 10^6 cells ml^-1) of treated and untreated mice were seeded into 96-well microplates (200 μl/well) and in vitro stimulated with SLA (10 μg
ml\(^{-1}\)) at 37°C for 48 h in a humid-saturated atmosphere containing 5% \(\text{CO}_2\). Lymphoproliferation was assessed by enumerating the cells using a hemocytometer [43]. Alternatively, for tracking lymphoproliferation by CFSE dilution, the SLA (10 \(\mu\)g ml\(^{-1}\))-stimulated lymphocytes (5 \times 10^6 cells ml\(^{-1}\)) from infected, treated and naïve mice were labeled with 1 \(\mu\)M CFSE for 48 h. After washing twice with PBS, the cells were resuspended in PBS and acquired in a BD LSR II flow cytometer to assess the population of cells that underwent proliferation. The contour plots were generated after appropriate gating [44].

Analysis of Th1/Th2 cytokines
Cytokine concentrations (IL-12, IL-4, IL-10 and IFN-\(\gamma\)) in the serum and splenocyte culture supernatants of differently treated mice were estimated by a multiplex bead-based assay as per the instructions of the manufacturer. [45–46]. Briefly, culture supernatants, serum samples and the cytokine standards were added in equal volumes to antibody-coated capture beads prior to incubation with biotinylated detection antibodies (anti-mouse) for 1 h at RT in the dark. The beads were washed with wash buffer (400 x g, 4°C, 5 min) and the supernatant gently aspirated. The beads were washed twice followed by incubation for a period of 1 h at RT in the dark with streptavidin-PE. After performing two additional centrifugation steps as described above, the beads were re-suspended in assay buffer and acquired on a BD LSR II flow cytometer (Becton Dickinson). The data were analysed with BD CBA software based on standard curves generated with recombinant cytokines.

Lymphocyte phenotyping
CD4\(^+\) and CD8\(^+\) T cell phenotyping was performed as previously described [47]. Splenocytes from treated and untreated BALB/c mice were co-stained with anti-CD4 FITC and anti-CD8 PE antibodies for 15 min on ice. The cells were then washed and resuspended in PBS for acquisition on a BD LSR II flow cytometer equipped with DIVA software.

Detection of intracellular IFN-\(\gamma\) levels
Intracellular detection of IFN-\(\gamma\)-producing CD4\(^+\) and CD8\(^+\) T lymphocytes was performed by flow cytometry. Single cell suspensions from spleens of treated and untreated infected mice were in vitro stimulated with 10 \(\mu\)g ml\(^{-1}\) SLA for 24 h and further incubated with Brefeldin A (10 \(\mu\)g ml\(^{-1}\)) for 1 h. After washing with FACS buffer, the cells were co-stained with APC and PE conjugated anti-CD4 and anti-CD8 antibodies, respectively. This was followed by washing, fixing and permeabilization with BD Cytofix/Cytoperm. The cells were then stained with FITC-anti-IFN-\(\gamma\) or isotype-matched control monoclonal antibodies (mAbs), and acquired and analyzed on a flow cytometer. The CD4\(^+\) and CD8\(^+\) T lymphocytes were gated individually and the expression of IFN-\(\gamma\)-producing cells ascertained [41].

Phenotypic analysis of co-stimulatory molecules (CD80 and CD86)
Peritoneal macrophages (2 \times 10^6 cells ml\(^{-1}\)) from infected BALB/c mice prior or subsequent to treatment were washed with FACS buffer (1X PBS with 1% FBS). To quantify the expression of co-stimulatory molecules, 2 \times 10^6 macrophages from each sample were stained with APC-labeled anti-CD80 (B7-1) and PE-Cy7 conjugated anti-CD86 (B7-2) mAbs on ice for 15 min. The cells were washed twice with PBS and flow cytometric acquisition was performed on BD LSR II equipped with DIVA software [48].
Analysis of memory CD8⁺ T cells
Splenocytes from infected BALB/c mice prior or subsequent to treatment were washed with FACS buffer and stained with anti-mouse CD8-APC, CD62L-PE and CD44-FITC (BD Pharmingen) for 30 min at 4°C, and then washed and fixed with 2% paraformaldehyde. Cells were acquired on a BD LSR II flow cytometer [49].

In vivo toxicity assay
Ten days post-treatment, blood was drawn from retro-orbital plexus of naïve, infected and treated BALB/c mice and serum separated. To evaluate the hepatic and renal functions, the serum levels of SGOT, SGPT, ALP, urea and creatinine were measured using commercially available kits (Span Diagnostics Ltd.) [39].

Statistical analyses
All the in vitro experiments were repeated at least twice. The in vivo data are from five mice per group. Statistical analysis was performed using Graph Pad Prism 5 software. P value was calculated using ANOVA with Tukey’s post-test. We considered P values <0.05 to be statistically significant. The graphs represent the mean with standard error bars.

Results
Eugenol nanoemulsion (EE)
The average droplet size and size distribution of eugenol nanoemulsion (EE) was found to be 990.8±2.64 nm and 0.23±0.01, respectively (Fig 2A).

Antileishmanial activity of EE against L. donovani in vitro and ex vivo
i) Determination of IC₅₀ of EE against promastigotes. The antileishmanial activity of EE was evaluated against L. donovani promastigotes by MTT reduction assay. EE (0–100 μg ml⁻¹) demonstrated dose-dependent killing of the promastigotes and IC₅₀ was achieved at 8.43 ±0.96 μg ml⁻¹ (Fig 2B). Parasite viability was not affected by the surfactant, T-80 (0.00175%) used as negative control (Fig 2F).

ii) Antileishmanial effect of EE against intracellular amastigotes. The activity of EE on internalized amastigotes was analyzed microscopically on Giemsa–stained macrophages. Our results depicted a dose-dependent inhibition of EE (0–100μg ml⁻¹) on amastigote infectivity with IC₅₀ of 5.05±1.72 μg ml⁻¹ (Fig 2C). At the highest dose of EE (100μg ml⁻¹) tested, there was almost complete clearance of the intracellular amastigotes (Fig 2C) as revealed from Giemsa-stained micrographs of parasitized macrophages. The amastigote infectivity was not affected by surfactant used as negative control (Fig 2F).

EE induced NO production ex vivo from parasitized macrophages
Low basal levels of NO were detected in the cell-free culture supernatants of infected macrophages, correlating with progression of disease. The NO release from infected macrophages upon subsequent incubation with EE (0–100 μg ml⁻¹) was dose-dependent (Fig 2D) and the levels were higher than that produced by normal macrophages. The highest dose of EE (100μg ml⁻¹) induced 14.56±1.16 and 20.03±3.28 μM of NO from normal and parasitized macrophages, respectively.
Fig 2. (A) Particle size distribution of eugenol emulsion (EE). Exponential phase *L. donovani* promastigotes were incubated at different concentrations of EE (0–100 μg/ml) as described in methods. (B) Peritoneal macrophages after infection, were incubated with increasing concentrations of EE (starting at 100 μg ml⁻¹) for 48 h at 37°C. (C) Intracellular amastigotes percentage (%) infectivity. (D & E) NO and superoxide anion generation by uninfected (filled circle) or infected (filled triangle) macrophages post-incubation with EE. Effect of Surfactant (F) on promastigotes, amastigotes and on RAW 264.7.
Cytotoxicity of EE on mammalian macrophages

Exposure of RAW 264.7 to EE (Fig 2E) and surfactant (Fig 2F) did not compromise the viability and morphology of the murine macrophage cell line.

Antileishmanial effect of EE in vivo

The intra-peritoneal administration of EE (75 mg/kg b.w.) for 10 consecutive days to 8-weeks infected BALB/c mice caused 87.01±5.85% (P<0.001) and 86.68±5.42% (P<0.001) decrease in parasitic load in spleen and liver, respectively (Fig 3A and 3B) at 10 days post-treatment. At lower dose of EE (50 mg/kg b.w.), 65.52±4.55% (P<0.001) and 61.19±7.76% (P<0.001) protection were conferred in spleen and liver, respectively. The lowest dose (25 mg/kg b.w.) resulted in more than 45% (P<0.001) fall in hepatic and splenic parasitic burden. AMB (5mg/kg b.w.) induced 92.22±4.96% and 94.88±4.25% elimination of parasites from liver and spleen, respectively. A significant reduction in spleen size (Fig 3C, inset) was also observed at 75mg/kg b.w. of EE, compared to the infected control. 50% and 25.68% reduction in spleen and liver weights, respectively was found with EE at this dose (Fig 3D and 3E), which was comparable to that obtained with AMB (52.33% and 28.41%).

EE induced leishmanial antigen-specific DTH

As an in vivo correlate of cell-mediated immunity, DTH reaction to FT was measured in infected mice at 10 days post-treatment. EE treatment resulted in a significant enhancement (P<0.001) in footpad thickness at 48 h compared with the control group (0.20±0.02 mm). A dose related increase in DTH reactivity was observed, with maximum swelling at 75 mg/kg b. w. (0.37±0.035 mm) followed by 50 and 25mg/kg b.w. (0.33±0.03 and 0.26±0.01 mm, respectively, Fig 4A). Whereas AMB treated mice showed marginal levels (0.27±0.02 mm) of DTH response.

Humoral immune response in EE-treated mice

At 10 days post-treatment, mice sera were assayed for FT-specific IgG1 and IgG2a isotype levels. IgG1 was detected at significantly (P<0.01) higher levels over IgG2a in infected control animals compared with the treated groups; the IgG2a:IgG1 ratio being 0.45 (Fig 4B). The highest IgG2a:IgG1 ratio (1.72) was observed in mice treated with 75mg/kg b.w. EE (P<0.001), followed by 50 mg/kg b.w. EE (1.28), AMB (1.14) and 25 mg/kg b.w. EE (0.97) treatment groups.

Effect of EE on NO production in protected mice

Host defense against intracellular pathogens including Leishmania is primarily mediated by NO and related RNIs [49]. NO production from peritoneal macrophages of EE treated mice was used to evaluate the effectiveness of EE on macrophage microbicidal activity. Nitrite, the stable end-product of NO metabolism was assessed by Griess reagent. Three-fold higher (9.54±0.77 μM) nitrite levels were found in mice treated with higher dose of EE (75mg/kg/b.w., P<0.001) as compared to infected control (3.78±0.63 μM) and the response was dose-dependent (Fig 4C). AMB treated mice induced moderate levels of nitrite (6.26±0.38 μM).
Lymphoproliferative effect of EE on the splenic and lymph node cells

The immunomodulatory potential of EE was evaluated through lymphoproliferation. Upon microscopic enumeration, a significant \((P < 0.001)\) proliferative effect of SLA-stimulated spleenocytes as well as lymphocytes was observed at 10 days post-EE treatment. The lymphoproliferative response was dose-dependent with maximum being elicited at 75mg/kg b.w. that was followed by 50 and 25mg/kg b.w. EE treatment In contrast, AMB did not induce significant SLA-specific lympho-proliferation (Fig 5A and 5B).

In parallel, the lymphoproliferative potential of lymphocytes following EE treatment was corroborated by CFSE staining. 28.16% and 28.02% lymphocytes from spleen and lymph nodes, respectively underwent cell division in normal mice. The lymphoproliferative response was the highest in spleen (78.7%) and lymph nodes (80.8%) upon treatment with EE (75 mg/
kg. b. w.). In untreated infected control group, we observed 29.28% and 28.14% lymphoproliferation in spleen and lymph nodes, respectively (Fig 5C and 5D).

**Effect of EE on Th1 and Th2 cytokine profile**

Changes in the levels of classical Th1 (IFN-γ and IL-2) and Th2 (IL-4 and IL-10) cytokines were assessed on day 10 post-treatment. Compared to untreated infected controls, EE (75mg/kg b.w.) induced enhanced serum levels of IFN-γ and IL-2 (2871±121 and 6943.5±129.77).
pgmL⁻¹, respectively, \( P \leq 0.001 \)) and significantly lower levels of IL-4 and IL-10 (472.5±96.53 and 182.5±30.76 pgmL⁻¹, \( P \leq 0.001 \)) (Fig 6A). Whereas, AMB treatment restored the cytokines to normal levels. Similar effect on Th1 and Th2 type cytokines was also observed with the culture supernatants of infected and treated mice (Fig 6B).

**EE induced expansion of CD4⁺ and CD8⁺ T cells:**
Mice with established \( L. \ donovani \) infection had low numbers of splenic CD4⁺ (7.2%) and CD8⁺ (4.1%) T cells (Fig 7) that enhanced to 14.4% and 7.6%, respectively 10 days post-treatment with EE (25 mg/kg b.w.). The T cell counts were increased with 50 mg/kg b.w. EE treatment (17% CD4⁺ and 9.4% CD8⁺ T cells). The CD4⁺ and CD8⁺ T cell population was, however, the highest (18.8% and 10.7%, respectively) at 75 mg/kg b.w. of EE (Fig 7A). These data support a bias towards Th1-driven effector functions and the role of CD4⁺ as well as CD8⁺ T cells in protection following EE treatment.

Fig 5. Effect of EE on SLA-specific lymphoproliferation in infected mice following therapy compared with the naïve and infected control. 10 days post-treatment, lymphocytes isolated from (A) spleen (5 \( \times \) 10⁶ cells mL⁻¹) and (B) lymph nodes (2 \( \times \) 10⁶ cells mL⁻¹) were plated aseptically and stimulated with SLA (10μg mL⁻¹) for 48 h and enumerated microscopically after trypan blue dye exclusion. In parallel, for lymphoproliferation by CFSE staining, lymphocytes isolated from (C) spleen and (D) lymph nodes were CFSE labeled (in triplicates) and stimulated with SLA for subsequent acquisition and analysis by FACS. Data represent the mean ± SE for five animals per group. Data were tested by ANOVA. Differences between means were assessed for statistical significance by Tukey’s test (*, \( P \leq 0.05 \); **, \( P \leq 0.01 \); ***, \( P \leq 0.001 \)). Results are from one of three representative experiments.

doi:10.1371/journal.pntd.0005011.g005
EE induced high frequency of IFN-γ secreting CD4 and CD8 T cells

CD4+ and CD8+ T cells are the main cellular sources of IFN-γ. In infected mice, low frequencies of IFN-γ-producing CD4+ (4.38±0.35%) and CD8+ (3.64±0.30%) T cells were detected which were upregulated by AMB treatment (9.04±0.72% CD4+, 6.89±0.36% CD8+). The maximum induction of IFN-γ-secreting CD4+ and CD8+ T cells occurred at 75mg/kg b.w. of EE (21.18±0.99%, 18.51±0.80%, respectively). While EE at 25mg/kg b.w. induced 9.02±1.06% CD4+ and 8.41±0.72% CD8+ T cells secreting IFN-γ that was comparable to that elicited by AMB (Fig 7B).

EE elicited CD80 and CD86 expression on APCs

The co-stimulatory molecules (CD80 and CD86) on APCs are essential for optimal T-APC talk and lymphocyte activation for secretion of effector cytokines. It was found that EE significantly augmented the expression of both CD80 and CD86 on peritoneal macrophages. EE (25 mg/kg b.w.) optimally boosted the number of double positive cells co-expressing CD80 and CD86 (27%) over the naive (15.5%) and infected control (12.9%) groups (Fig 8). The response was dose-dependent with heightened expression at 50 mg/kg b.w. of EE (28.9%). CD80 and CD86 double positive cells were maximally expressed at 75 mg/kg b.w. EE (32.5%). The response in case of AMB treated group was also enhanced up to a moderate level (Fig 8).
Generation of memory CD8 T (CD44+CD62L+) lymphocytes

Memory T cell generation in the host is indicative of resistance to *Leishmania* re-infection [50]. The subtle co-expression of CD44 and CD62L (10.7%) in the infected control group, was up-regulated (16.73%) upon treatment with EE at 75mg/kg b.w., in concordance with resolution of disease and generation of central memory cells. AMB had insignificant effect (10.93%) on induction of memory T cells (Fig 9).

**In vivo** toxicity of EE by biochemical analysis of serum enzymes:

Liver function (ALP, SGOT and SGPT) and renal function tests (urea and creatinine) were done 10 days post-administration of EE in naive BALB/c mice (Table 1) as well as infected mice upon treatment (Table 2). EE treated group showed normal values of serum enzymes (up to 75 mg/kg b.w.), indicating no *in vivo* toxicity.

**Discussion**

The immune system is known to synergistically augment the therapeutic effectualness of antiparasitic drugs [51]. The administration of immunomodulators in conjunction with conventional chemotherapy to rejuvenate the host immune response has a multitude of benefits and aids in advancing the current therapeutic effectiveness by reducing the dose and duration of treatment and hence the toxicity of the drugs. Thus, antileishmanial drugs that can concomitantly ameliorate the immune suppression of the infected hosts, have a two-prong effect and are sought after [52–56].
Previous report showed that the essential oil of *S. aromaticum* (EROSA), mediated programmed cell death against *L. donovani* without affecting the host macrophages. Eugenol was the major constituent in EROSA [31]. This is the first report of therapeutic and immunostimulatory potential of emulsion of eugenol (EE) against experimental VL. EE showed profound antileishmanial efficacy, coupled with cell-mediated immunopotentiation without any adverse effect on the host. Furthermore, EE exhibited a concentration-dependent significant antileishmanial activity against *L. donovani* promastigotes as well as the intramacrophagic amastigotes.
Ex vivo studies revealed non-toxicity of EE against murine macrophages even at 100μg ml⁻¹. In case of L. amazonensis, clearance of both axenic and intracellular amastigotes has been attributed to NO [57]. We observed a significant increase in NO (20.03±3.28 μM) post-incubation of amastigote-infested macrophages with EE, harmonizing with the previous reports. The EE-induced NO production from infected macrophages strengthened the role of RNS in amastigote death.

Intra-peritoneal treatment with EE significantly (P<0.001) lowered the splenic and hepatic parasite loads in infected mice to levels similar to that achieved with AMB. This was coupled with restoration of liver and spleen weights to normal levels. Our results warrant further studies with lower doses of EE.

Our study corroborates immunomodulation complementing the leishmanicidal effect of EE, apparently through Th1 cytokine-driven and macrophage-mediated mechanisms which are also NO-dependent. Immunostimulatory effects have been reported for several natural products, offering a rational basis for their therapeutic potency. In case of leishmaniasis, an ideal drug is envisaged to have dual effects—to directly and selectively kill the parasites as well as rejuvenate the depressed immunity towards Th1 bias [58, 55].

Fig 9. Analysis of CD62L and CD44 expressing memory subsets on splenic lymphocytes isolated from differently treated and infected mice. 2 x 10⁶ lymphocytes were stained with anti-CD8-APC, anti-CD44-FITC and anti-CD62L-PE. Cells were gated on CD8⁺ T lymphocytes. Comparison between the infected vs. EE treated groups is shown. Data represent the mean ± SE for five animals per group. *, P ≤ 0.05; **, P ≤ 0.01; ***, P ≤ 0.001 compared with the control group determined by one-way ANOVA. Results are from one of three representative experiments.

doi:10.1371/journal.pntd.0005011.g009

Table 1. Effect of EE (higher dose) on hepatic and renal functions of naive BALB/c mice

| Group (n = 5) | SGOT (U/L) | SGPT (U/L) | ALP (U/L) | Urea (mg/dL) | Creatinine (mg/dL) |
|--------------|-----------|-----------|-----------|--------------|-------------------|
| Control      | 55.40±5.12| 32.09±10.01| 85.06±1.37| 16.10±2.15  | 0.86±0.14         |
| EE 75 mg/kg (i.p) | 52.9±3.19 | 30.98±7.36 | 84.16±6.06| 15.49±3.61  | 0.85±0.08         |

Mice (n = 5) received EE for 10 consecutive days. Enzyme estimations (means: SE) were done using commercial kits.

doi:10.1371/journal.pntd.0005011.t001
During the progression of leishmaniasis, cytokine milieu switches from Th1 to Th2 profile [59–60]. An effective therapeutic intervention against *L. donovani* entails mounting of a strong Th1 response. To prove that EE has immunomodulatory effect, we analysed the cytokines in serum and culture supernatants of lymphocytes from infected BALB/c mice 10 days post-treatment. Therapy with EE mounted a polarized Th1 response with enhanced IFN-γ and IL-2 secretion, which stimulated microbicidal responses of macrophages leading to NO production that was demonstrated in this study *ex vivo* as well as *in vivo*. Both reactive oxygen and nitrogen species have been implicated to contribute to reduction in parasitism *ex vivo* as well as *in vivo*, and suppression of either compromises the macrophage-leishmanicidal activity [61–62]. IFN-γ is known to stimulate iNOS2 expression with significant NO secretion, that may have provided impressive levels of protection. In our studies, the antagonistic effect of IFN-γ in reducing Th2-associated cytokines, IL-10 and IL-4 was also found in treated mice. Detailed immunological analysis of infected mice depicted persistent IgG1 levels, probably maintained by IL-4 secretion [63]. Treatment with EE was associated with increased IgG2a, possibly due to up-regulated serum levels of IFN-γ. Plant-derived natural products have been found to exert leishmanicidal activity via modulation of the Th1/Th2 responses. Quassin inhibited *L. donovani* growth by switching from Th2 (IL-4 and IL-10) to Th1 (IFN-γ and IL-2) cytokine [21]. A similar effect occurred with EE, which enhanced IFN-γ and NO production, coupled with marked reduction in the *L. donovani* load in infected macrophages.

EE induced a marked switch in *L. donovani*-infected BALB/c mice from disease-promoting to *Leishmania*-specific disease-resolving humoral as well as cell-mediated immune responses. EE at 75mg/kg b.w. rescued T-cell-anergic conditions, inducing elevated levels of DTH, lymphoproliferation, IL-2, IFN-γ and NO, and maximally reduced the Th1 suppressive cytokines (IL-4 and IL-10), in a dose-dependent manner, whereas the immune response was restored following treatment with AMB (5mg/kg/b.w).

VL is associated with impaired immunological responses. Effectual antileishmanial therapy entails robust cellular immune responses in addition to antibodies. Experimental models of VL have shown that CD8+ T cells are instrumental in controlling *L. donovani/L. infantum* infection, through their ability to secrete IFN-γ and/or their cytolytic activity [64]. Furthermore, CD8+ coupled with CD4+ T cells, are essential to thwart reactivation of murine VL, the present investigation showed that the splenic CD4+ and CD8+ T lymphocyte counts were significantly boosted by administration of EE (75mg/kg b.w.) to *L. donovani* infected BALB/c mice as compared with untreated infected controls. The therapeutic significance of EE in VL was strengthened by the development of central memory (CD62Lhigh CD44high) CD8+ T lymphocytes.

T cells are known to interact with APCs and play vital roles in eliminating pathogens resident within macrophages. For optimal activation of naive T cells, the T-cell receptor (TCR)
interacts with the peptide-MHC complex presented by professional APCs, while the second signal is delivered by co-stimulatory molecules, and the secretion of pro-inflammatory cytokines [65]. The ligation of CD80 and/or CD86 on APCs with CD28 on T cells induces the DCs to secrete IL-6 and IFN-γ for optimal T and B cell activation, proliferation, and differentiation [66]. There are reports indicating down modulation of CD80 and 86 expression in certain diseases [67–68], including leishmaniasis. In our studies, the expression of both CD80 and CD86 on peritoneal macrophages of *L. donovani* infected mice were found to be significantly enhanced upon treatment with EE, substantiating their immunomodulatory potency. Our results indicate the potentiality of EE in activating the T cells through upregulation of co-stimulatory signals that help to mount an effective immune response by secreting Th1 cytokines such as IFN-γ and IL-2.

The deterioration in renal and hepatic functions is the major dose-limiting side effect of currently available chemotherapeutic drugs. In the present study, upon administration of EE (25, 50 and 75mg/kg b.w.) to naïve and *L. donovani* infected BALB/c mice, the serum levels of SGOT, SGPT, ALP, urea and creatinine were found to be in the normal range, proving absence of hepato- and nephro-toxicity.

In conclusion, our findings highlight the *in vitro* and *ex vivo* leishmanicidal effect of EE that occurred via an increase in production of NO without adverse effects on mammalian macrophages. On the other hand, treatment of infected mice with EE significantly waned the hepatic...
and splenic parasite loads with diminution in spleen and liver weights. EE induced enhanced lymphoproliferation, up-regulated co-stimulatory molecules (CD80/CD86) expression on APCs, and resulted in expansion of CD4^+ and CD8^+ T cell numbers and generation of central memory cells. EE further suppressed Th2 cytokines (IL-4 and IL-10) and stimulated the production of Th1 cytokines (IFN-γ and IL-2) with release of NO from the peritoneal macrophages. Th1-driven immune polarization was also reflected from high IgG2a/low IgG1 ratio and significant elicitation of DTH response. Thus, protection against *L. donovani* infection in the EE treated animals was due to direct parasite killing as well as the induction of cellular immunity via immunopotentiation as depicted in Fig 10.

**Acknowledgments**

We are obliged to Dr. Mohd. Aslam for support in the initial phase of this study.

**Author Contributions**

Conceived and designed the experiments: MI FA.

Performed the experiments: MI GC FA.

Analyzed the data: MI FA.

Contributed reagents/materials/analysis tools: FA HAO HAH.

Wrote the paper: MI FA HAO HAH.

Assisted in ex vivo experiments: GC MYW. Contributed to reviewing of manuscript: FA HAO HAH.

**References**

1. Wilson ME, Jeronimo SM, Pearson RD (2005) Immuno-pathogenesis of infection with the visceralizing *Leishmania* species. Microb Pathog 38: 147–160. doi: 10.1016/j.micpath.2004.11.002 PMID: 15797810
2. Kaye P, Scott P (2011) Leishmaniasis: Complexity at the host-pathogen interface. Nat Rev Micro 9: 604–615.
3. Sacks D, Noben-Trauth N (2002) The immunology of susceptibility and resistance to *Leishmania major* in mice. Nat Rev Immunol 2: 845–858. doi: 10.1038/nri933 PMID: 12415308
4. Rodrigues V, Laforge M, Campillo-Gimenez L, Soundaramoury C, Correia-de-Oliveira A, Dinis-Oliveira RJ, et al. (2014) Abortive T Follicular Helper Development Is Associated with a Defective Humoral Response in *Leishmania infantum*-Infected Macaques. PLoS Pathog 10(4): e1004096. doi: 10.1371/journal.ppat.1004096 PMID: 24763747
5. Nylen S, Maurya R, Eidsmo L, Manandhar KD, Sundar S, Sacks D (2007) Splenic accumulation of IL-10 mRNA in T cells distinct from CD4+CD25+ (Foxp3) regulatory T cells in human visceral leishmaniasis. J Exp Med 204: 805–817. doi: 10.1084/jem.20061141 PMID: 17389235
6. Peters N, Sacks D 2006 Immune privilege in sites of chronic infection: *Leishmania* and regulatory T cells. Immunol Rev 213: 159–179. doi: 10.1111/j.1600-065X.2006.00432.x PMID: 16972903
7. Myler P, Fasel N 2008 *Leishmania*: After the genome. Caister Academic Press ISBN 978-1-904455-28-8.
8. Nazmul (2007) Drug resistance in visceral leishmaniasis. J Medicine 8: 37–38.
9. Dantas Torres F (2006) Leishmune vaccine: the newest tool for prevention and control of canine visceral leishmaniosis and its potential as a transmission-blocking vaccine. Vet Parasitol 141: 1–8. doi: 10.1016/j.vetpar.2006.05.001 PMID: 16750885
10. Croft SL, Coombs GH (2003) Leishmaniasis: current chemotherapy and recent advances in the search for novel drugs. Trends Parasitol 19: 502–508. PMID: 14580961
11. Croft SL, Sundar S, Fairlamb AH (2006) Drug resistance in leishmaniasis. Clin Microbiol Rev 19: 111–126. doi: 10.1128/CMR.19.1.111-126.2006 PMID: 16418526
12. Eiras DP, Kirkman LA, Murray HW (2015). Cutaneous Leishmaniasis: Current Treatment Practices in the USA for Returning Travelers. Curr Treat Options Infect Dis 7: 52–62. doi: 10.1007/s40506-015-0038-4 PMID: 25788870

13. Akendengue B, Ngou-Mila ma E, Leurens A, Hocquemer R (1999) Recent advances in the fight against leishmaniasis with natural products. Parasite 6: 3–8. PMID: 10229931

14. Kayser O, Kiderlen AF, Croft SL (2003) Natural products as anti-parasitic drugs. Parasitol Res 90: 55–62.

15. Passero LF, Laurenti MD, Santos-Gomes G (2014) Plants used in traditional medicine: extracts and secondary metabolites exhibiting antileishmanial activity. Curr Clin Pharmacol 9: 187–204. PMID: 23173968

16. da Costa-Silva TA, Grecco SS, de Sousa FS, Lago JH, Martins EG, Terrazas CA, Varikuti S, Owens KL, Beverley SM, Satoskar AR, Tempone AG (2015) Immunomodulatory and Antileishmanial Activity of Phenylpropanoid Dimers Isolated from Nectandra leucantha. J Nat Prod 78: 653–657 doi: 10.1021/np500809a PMID: 25835647

17. Pourrajab F, Forouzannia SK, Tabatabae SA (2012) Novel immunomodulatory function of 1,3,4-thia-diazole derivatives with leishmanicidal activity. J Antimicrob Chemother 67:1968–1978. doi: 10.1093/jac/dks144 PMID: 22581907

18. Neris PL, Calsdas JP, Rodrigues YK, Amorim FM, Leite JA, Rodrigues-Mascarenhas S, et al. (2013) Neolignan Licarin A presents effect against Leishmania major associated with immunomodulation in vitro. Exp Parasitol 135: 307–313 doi: 10.1016/j.exppara.2013.07.007 PMID: 23891431

19. Chowdhury S, Mukherjee T, Mukhopadhye R, Mukherjee B, Sengupta S, Chattopadhyay S, et al. (2012) The lignan niranthin poisons Leishmania donovani/topoisomerase IB and favours a Th1 immune response in mice. EMBO Mol Med 4: 1126–1143. doi: 10.1002/emmm.2011021316 PMID: 23027614

20. Dos Santos RA, Batista J Jr, Rosa SI, Torquato HF, Bassi CL, Ribeiro TA, et al. (2011) Leishmanicidal effect of Spiranthera odoratissima (Rutaceae) and its isolated alkaloid skimmi anine occurs by a nitric oxide dependent mechanism. Parasitology 138: 1224–1233. doi: 10.1016/S0031-1820(11)00116-8 PMID: 21810308

21. Bhattacharjee S, Gupta G, Bhattacharya P, Mukherjee A, Mujumdar SB, Pal A, et al. (2009) Quassin alters the immunological patterns of murine macrophages through generation of nitric oxide to exert antileishmanial activity. J Antimicrob Chemother 63: 317–324. doi: 10.1093/jac/dkn479 PMID: 19036753

22. Kolodziej H, Kiderlen AF (2005) Antileishmanial activity and immune modulatory effects of tannins and related compounds on Leishmania parasitised RAW 264.7 cells. Phytochemistry 66: 2056–2071. doi: 10.1016/j.phytochem.2005.01.011 PMID: 16153409

23. Shafyekh N, Sane SA, Gupta S (2011) Antileishmanial efficacy of fluconazole and miltefosine in combination with an immunomodulator picroliv. Parasitol Res 108: 793–800. doi: 10.1007/s00436-010-2230-2 PMID: 21212890

24. Rosa MSS, Mendonca a-Filho RR, Bizzo HR, Rodrigues IA, Soares RM, Souto-Padron T (2003) Antileishmanial activity of a linalool-rich essential oil from Croton cajucara. Antimicrob Agents Chemother 47: 1895–1901. doi: 10.1128/AAC.47.6.1895-1901.2003 PMID: 12766084

25. Chouhan G, Islamuddin M, Sahal D, Afrin F (2014) Exploring the role of medicinal plant-based immunomodulators for effective therapy of leishmaniasis. Front Immunol 5:193. doi: 10.3389/fimmu.2014.00193 PMID: 24829566

26. Mishra RK, Singh SK (2008) Safety assessment of Syzygium aromaticum flower bud (clove) with respect to testicular function in mice. Food Chem Toxicol 46: 3333–3338. doi: 10.1016/j.fct.2008.08.006 PMID: 18765266

27. Burt SA, Reinders RD (2003) Antibacterial activity of selected plant essential oils against Escherichia coli O157: H7. Lett Appl Microbiol 36: 162–167. PMID: 12581376

28. Santoro GF, Cardoso MG, Guimaraes LG, Mendonça LZ, Soares MJ (2007) Trypanosoma cruzi: activity of essential oils from Achille amellifolium L., Syzygium aromaticum L. and Ocimum basilicum L. on epimastigotes and trypomastigotes. Exp Parasitol 116: 283–290. doi: 10.1016/j.exppara.2007.01.018 PMID: 17349626

29. Bagavan A, Rahuman AA, Kaushik NK, Sahal D (2011) In vitro antimarial activity of medicinal plant extracts against Plasmodium falciparum. Parasitol Res 108: 15–22. doi: 10.1007/s00436-010-2034-4 PMID: 20889417

30. Carrasco FR, Schmidt G, Romero AL, Santoretto JL, Caparroz-Assef SM, Bersani-Amado CA, et al. (2009) Immunomodulatory activity of Zingiber officinale, Salvia officinalis L. and Syzygium aromaticum L. essential oils: evidence for humor- and cell-mediated responses. J Pharm Pharmacol 61: 961–967. doi: 10.1211/jpp/61.07.0017 PMID: 19589240
31. Islamuddin M, Sahal D, Afirn F (2014) Apoptosis-like death in Leishmania donovani/promastigotes induced by eugenol-rich oil of Syzygium aromaticum. J Med Microbiol 63: 74–85. doi: 10.1099/jmm.0.064709-0 PMID: 2416990

32. Luo Y, Zhang Y, Pan K, Critzer F, Davidson PM, Zhong Q (2014) Self-emulsification of alkaline-dissolved clove bud oil by whey protein, gum arabic, lecithin, and their combinations. J Agric Food Chem 62:4417–4424. doi: 10.1021/jf500698k PMID: 24758517

33. Kamel AA, Alexei RK (2015) Eugenol oil nanoemulsion: antifungal activity against Fusarium oxy-sporum f. sp. vasinfectum and phytotoxicity on cottonseeds. Appl Nanosci 5: 255–265.

34. Hamed SF, Sadek Z, Edris A (2012) Antioxidant and antimicrobial activities of clove bud essential oil and eugenol nanoparticles in alcohol-free microemulsion. J Oleo Sci 61: 641–648. PMID: 23138253

35. Das R, Roy A, Dutta N (2008) Reactive oxygen species and imbalance of calcium homeostasis contributes to curcumin induced programmed cell death in Leishmania donovani. Apoptosis 13: 867–882. doi: 10.1007/s10495-008-0224-7 PMID: 18506627

36. Varela MRE, Villa-Pulgarin JA, Yepes E, Muller I, Modolell M, Munoz DL, et al. (2012) In vitro and in vivo efficacy of etherlipid edelfosine against Leishmania spp. And SbV-resistant parasites. PLoS Negl Trop Dis 6:e1612. doi: 10.1371/journal.pntd.0001612 PMID: 22506086

37. Dutta A, Sarkar D, Gurib-Fakim A, Mandal C, Chatterjee M (2008) In vitro and in vivo activity of Aloe vera leaf exudates in experimental visceral leishmaniasis. Parasitol Res 102: 1235–1242. doi: 10.1007/s00436-008-0899-2 PMID: 18266009

38. Abdian N, Golhami E, Zahedifard F, Safaei N, Rafati S (2011) Evaluation of DNA/DNA and prime-boost vaccination using LPG3 against Leishmania major infection in susceptible BALB/c mice and its antigenic properties in human leishmaniasis. Exp Parasitol 127: 627–636. doi: 10.1016/j.exppara.2010.12.007 PMID: 21187087

39. Islamuddin M, Chouhan G, Farooque A, Dwarakanath BS, Sahal D, Afirn F (2015) Th1-Biased Immuno modulation and Therapeutic Potential of Artemisia annua in Murine Visceral Leishmaniasis. PLoS Negl Trop Dis 9(1): e3321. doi: 10.1371/journal.pntd.0003321 PMID: 25568967

40. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. (1951) Protein measurement with the Folin phenol reagent. J Biol Chem 193: 265–275. PMID: 14907713

41. Banerjee A, De M, Ali N (2011) Combination Therapy with Paromomycin-Associated Stearylamine-Bearing Liposomes Cures Experimental Visceral Leishmaniasis through Th1-Biased Immunomodulation. Antimicrob Agents Chemother 55: 1661–1670. doi: 10.1128/AAC.00524-10 PMID: 21220536

42. Wang YP, Li XY (2002) Effect of astra galoside IV on T, B lymphocyte proliferation and peritoneal macrophage function in mice. Acta Pharmacol Sin 3: 263–266.

43. Seo YC, Choi WY, Lee CG, Cha SW, Kim YO, Kim JC, et al. (2011) Enhanced immunomodulatory activity of Gelatin-Encapsulated Rubus coreanus Miquel Nanoparticles. Int J Mol Sci 12: 9031–9056. doi: 10.3390/ijms12129931 PMID: 22272118

44. Del-Rey MJ, Manzanares J, Bosque A, Aguilo JI, Gomez-Rial J, Roldan E, et al. (2007) Auto-immune lymphoproliferative syndrome (ALPS) in a patient with a new germline Fas gene mutation. Immunobiology 212: 73–83. doi: 10.1016/j.imbio.2006.12.003 PMID: 17336828

45. Ding AH, Nathan CF, Stuehr DJ (1988) Release of reactive nitrogen intermediates and reactive oxygen intermediates from mouse peritoneal macrophages: comparison of activating cytokines and evidence for independent production. J Immunol 141: 2407–2412. PMID: 319757

46. Agarwal V, Sharma AK, Upadhyay A, Singh G, Gupta R (2012) Hypoglycemic effects of Citrullus colocynthis roots. Acta Pol Pharm 69: 65–79. PMID: 22574509

47. Gupta A, Khajuria A, Singh J, Bedi KL, Satti NK, Dutt P, et al. (2006) Immunomodulatory activity of biopolymeric fraction RLJ-NE-205 from Picrorhiza kurroa. Int Immunopharmacol 6: 1543–1549. doi: 10.1016/j.imppharm.2006.05.002 PMID: 16919826

48. Khan S, Malik F, Suri KA (2009) Molecular insight into the immune up-regulatory properties of the leaf extract of Ashwagandha and identification of Th1 immuno stimulatory chemical entity. Vaccine 27: 6080–6087. doi: 10.1016/j.vaccine.2009.07.011 PMID: 19628058

49. Bogdan C, Rollinghoff M (1998) The immune response to Leishmania: mechanisms of parasite control and evasion. Int J Parasitol 28: 121–134. PMID: 9504340

50. Zaph C, Uzonova J, Beverley SM, Scott P (2004) Central memory T cells mediate long-term immunity to Leishmania major in the absence of persistent parasites. Nature Medicine 10: 1104–1110. doi: 10.1038/nm1108 PMID: 15448686

51. Berger BJ, Fairlamb AH (1992) Interactions between immunity and chemotherapy in the treatment of trypanosomiasis and leishmaniasis. Parasitology 105: 71–78. PMID: 1308932
52. Firdessa R, Good L, Amstalden MC, Chindera K, Kamaruzzaman NF, Schultheis M, et al. (2015) Pathogen- and Host-Directed Antileishmanial Effects Mediated by Polyhexanide (PHMB). PLoS Negl Trop Dis 9(10): e0004041. doi: 10.1371/journal.pntd.0004041

53. da Costa-Silva TA, Grecco SS, de Sousa FS, Lago JHG, Martins EGA, Terrazas CA, Varikuti S, Owens KL, Beverley SM, Satoskar AR, Tempone AG (2015) Immunomodulatory and Antileishmanial Activity of Phenylpropanoid Dimers Isolated from Nectandra leucantha. J Nat Prod. 78: 653–657. doi: 10.1021/np500809a PMID: 25835647

54. Sharma M, Sehgal R, Kaur S (2012) Evaluation of Nephroprotective and Immunomodulatory Activities of Antioxidants in Combination with Cisplatin against Murine Visceral Leishmaniasis. PLoS Negl Trop Dis 6(5): e1629. doi: 10.1371/journal.pntd.0001629 PMID: 22563510

55. Banerjee A, De M, Ali N. (2011) Combination therapy with paromomycin-associated stearylamine-bearing liposomes cures experimental visceral leishmaniasis through Th1-biased immunomodulation. Antimicrob Agents Chemother. 55:1661–1670. doi: 10.1128/AAC.00524-10 PMID: 21220536

56. Barroso PA, Marco JD, Calvopina M, Kato H, Korenaga M, Hashiguchi Y (2007) A trial of immunotherapy against Leishmania amazonensis infection in vitro and in vivo with Z-100, a polysaccharide obtained from Mycobacterium tuberculosis, alone or combined with meglumine antimoniate. J Antimicrob Chemother. 59:1123–1129. doi: 10.1093/jac/dkm079 PMID: 17439977

57. Holzmuller P, Sereno D, Cavaleyra M, Mangot I, Daulouede S, Vincenteau P, et al. (2002) Nitric oxide mediated proteasome dependent oligonucleosomal DNA fragmentation in Leishmania amazonensis amastigotes. Infect Immun 70: 3727–3735. doi: 10.1128/IAI.70.7.3727-3735.2002 PMID: 12065515

58. Herbert KA, Albrecht FK (2005) Antileishmanial activity and immune modulatory effects of tannins and related compounds on Leishmania parasitized RAW 264.7 cells. Phytochemistry 66: 2056–2071. doi: 10.1016/j.phytochem.2005.01.011 PMID: 16153409

59. Awasthi A, Mathur RK, Saha B (2004) Immune response to Leishmania infection. Indian J Med Res 119: 238–258 PMID: 15243162

60. Kemp M, Kurtzhals JA, Bendtzen K, Poulsen LK, Hansen MB, Koech DK, et al. (1993) Leishmania donovani reactive Th1- and Th2 like cell clones from individuals who have recovered from visceral leishmaniasis. Infect Immun 61: 1069–1073. PMID: 8432588

61. Polley R, Stager S, Prickett S, Maroof A, Zubairi S, Smith DF, et al. (2006) Adoptive immunotherapy against experimental visceral leishmaniasis with CD8+ T cells requires the presence of cognate antigen. Infect Immun 74: 773–776. doi: 10.1128/IAI.74.1.773-776.2006 PMID: 16399038

62. Curtsinger JM, Mescher MF (2010) Inflammatory cytokines as a third signal for T cell activation. Curr Opin Immunol 22: 333–340. doi: 10.1016/j.coi.2010.02.013 PMID: 20393604

63. Gowthaman U, Chodisetti SB, Agrewala JN (2010) T cell help to B cells in germinal centers: putting the jigsaw together. Int Rev Immunol 29: 403–420. doi: 10.3109/08830185.2010.496503 PMID: 20635881

64. Sun P, Fernandez S, Marovich MA, Palmer DR, Celluzzi CM, Boonnak K, et al. (2009) Functional characterization of ex vivo blood myeloid and plasmacytoid dendritic cells after infection with dengue virus. Virology 383: 207–215. doi: 10.1016/j.virol.2008.10.022 PMID: 19013627

65. Libraty DH, Pichyangkul S, Ajariyakhajorn, Endy TP, Ennis FA (2001) Human dendritic cells are activated by dengue virus infection: enhancement by gamma interferon and implications for disease pathogenesis. J Virol 75: 3501–3508. doi: 10.1128/JVI.75.8.3501-3508.2001 PMID: 11264339