

A brief report on eugenol oleate is an oral immunomodulatory molecule against visceral leishmaniasis

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ABSTRACT

Visceral Leishmaniasis (VL) is a life-threatening infectious disease and is acclaimed as a neglected tropical disease, caused by *Leishmania donovani*. It leads to severe immune suppression in the host defense system. Higher cytotoxicity, rigorous side effects, and lower therapeutic indexes (TI) of current antileishmanial drugs have created a necessity to develop new molecules with better antileishmanial activity and high TI value. We synthesized a plenty number of derivatives of Eugenol and screened them for their activity against promastigote and amastigote forms of *L. donovani*. Among the synthesized derivatives, Eugenol oleate showed better antileishmanial activity against extracellular promastigotes (IC_{50} - $20.13 \pm 0.91 \mu\text{M}$) and intracellular amastigotes (EC_{50} - $4.25 \pm 0.26 \mu\text{M}$). The TI value (82.24 ± 3.77) was found to improve by 10 to 13-folds compared to amphotericin B and miltefosine, respectively. Treatment with Eugenol oleate activated the PKC- β II-p38 MAPK and produced IL-12 and IFN- γ , which activated the iNOS2 to produce NO free radicals that cleared the intracellular parasite. Eugenol oleate was found safe with an appreciable pharmacokinetic profile for the oral anti-VL agent. This molecule increased the Th1 cytokine profile and decreased the Th2 cytokine profile observed from ELISA and qPCR, suggesting that the Eugenol oleate induced the parasite clearance through the activation of the host immune system. Hence, this study represented that Eugenol oleate, an oral immunomodulatory molecule, could induce a host protective immune response against visceral leishmaniasis through enhanced NO generation and Th1 response, essential against this deadly disease.

Keywords: Eugenol oleate; Oral administration; Visceral leishmaniasis; Immunomodulation.

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INTRODUCTION

A lack of new anti-parasitic medications and restricted chemotherapeutic choices may make innovative therapies that target host immunity an effective choice in the present era. In recent years, drug resistance in Visceral Leishmaniasis (VL) has become a serious barrier to effective therapy. In the current context, immunomodulators are essential to combating numerous diseases.¹ Immunomodulators strengthen the immune response against infectious diseases and cancers.²⁻⁵ It has been demonstrated that several immunomodulators have antileishmanial properties.⁶⁻¹⁰ Immunomodulators tested as antileishmanial lead frequently include glycyrrhizic acid (GA), arabinosylated lipoarabinomannan (Ara-LAM), and sodium antimony gluconate (SAG).¹⁰⁻¹² Using immunomodulators resulted in the down-regulation of PKC ζ , the conversion of anti-inflammatory cytokines into pro-inflammatory cytokines, and the inhibition of plasma-membrane-calcium-ATPase in infected macrophages, according to a more recent study by Roy *et al.*¹³ Many phytochemicals produced from plants and their chemically manufactured derivatives may offer a novel therapeutic strategy in this situation. In this context, few reports indicated that simple chemical modification of Eugenol had improved its anti-VL efficacy.¹⁴ In this study, we have synthesized derivatives of Eugenol and screened them for their activity against promastigote and amastigote forms of *Leishmania donovani*, the causative parasite of VL.

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MATERIALS AND METHODS

Chemical and reagents

Fetal bovine serum (FBS) and the antibiotics were bought from Gibco BRL (Grand Island, NY, USA). We purchased the Quantikine M ELISA Kit for IFN- γ , IL-12, TNF- α , IL-2, and IL-10 from R & D Systems in the USA. The cDNA synthesis kit and the RT-PCR chemicals were bought from Fermentas and TAKARA Bio. All the primary antibodies were from Santa Cruz Biotechnology, Inc. (USA), and the secondary antibodies were from Sigma (USA). The finest grade of all other chemicals was obtained from Sigma (USA), Merck-Milipore (India), and Himedia (India).

Animals, parasite, and ethics statement

BALB/c mice were purchased from SASTRA Deemed University's central animal facility in Thanjavur. The virulence of *L. donovani* (MHOM/IN/AG/83) was maintained by passage through BALB/c mice with i.v. Infection of stationary phase promastigotes (2×10^7 /mice). This was done in vitro using RPMI1640 medium (Gibco), supplemented with 10% FBS (Gibco), and 1X penicillin-streptomycin (Gibco). The "Guide for the Care and Use of Laboratory Animals of the National Institutes of Health" guidelines were strictly followed during this investigation. The Institutional Animal Ethics Committee gave its prior clearance to each experimental animal procedure (SASTRA Deemed University, Registration Number: 817/PO/ReRc/S/04/ CPCSEA; Dated: 20.11.2015).

Synthesis of Eugenol oleate

In the laboratory, Eugenol oleate was produced, as was previously reported, and confirmed by ^1H NMR.¹⁵

In vitro Amastigote and Promastigote killing assay

BALB/c mice were injected with 4% starch and sacrificed after 48 h to collect peritoneal lavage, which was cultured in RPMI-1640 medium with 10% FBS and antibiotics for 48 hours for further experiments.^{15,16} On 8 well chamber glass slides from Genetix, mouse (BALB/c) peritoneal macrophages were cultivated at a cell density of 1×10^5 cells per 200 μL of RPMI1640 supplemented with 10% FBS. Promastigotes were introduced into peritoneal macrophages at a ratio of 1:10 for 6 hours at 37°C with 5% CO_2 . Promastigotes that weren't internalized were eliminated by washing twice with RPMI-1640 medium. Thereafter, for 1-hour, cells were either treated or left untreated with the inhibitors L-NMMA (0.4 mM), NAC (10 mM), PKC- inhibitor peptide (6 M), and p38-inhibitor SB203580 (5 g/mL), and Eugenol oleate (2.5 g/mL) was applied for 48 hours.^{15,17,18} Macrophages were then preserved in ice-cold methanol and stained with Giemsa dye after being rinsed with 1X PBS. Using a light microscope the parasite burden was estimated as the number of amastigotes/100 macrophages.^{15,19}

The MTT assay was used to determine the anti-promastigote activity. Promastigotes were seeded into 96-well flat bottom plates (Genetix) at a density of 1×10^6 parasites per well in a final volume of 200 μL media, and they were then cultured with Eugenol derivatives made synthetically (0–250 mg/mL) or known antileishmanial at escalating doses 48 hours of parasite incubation at 22°C was followed by an MTT assay. Using a microplate reader absorbance values at 570 nm were calculated (Biotek, Synergy H1). Promastigotes viability was calculated as a percentage compared to control sets that weren't treated.^{15,21}

Plasma pharmacokinetic and acute toxicity study

The test substance, eugenol oleate, was given orally to BALB/c mice (8–9 weeks old) on a fasted state in a vehicle (dimethylacetamide + tween 80 + sterile water) at a dose

volume of 10 mL/kg B.W. Blood samples were taken under mild isoflurane anesthesia using the retro-orbital puncture method to draw blood into pre-labeled tubes containing an anticoagulant (K2EDTA-2 mg/mL blood) at regular intervals following dose. Plasma was isolated from collected blood samples by centrifugation, and it was then kept at -80°C pending analysis. Eugenol oleate was examined in the plasma samples using HPLC. The plasma pharmacokinetics (PK) parameters, including C_{max} , T_{max} , AUC_{last} , AUC_{inf} , $T_{1/2}$, and MRT last were calculated from the non-compartmental analysis using the computer program WinNonlin (Pharsight Corporation, California).²⁰ Acute toxicity for the limit test was conducted following the recommendations for testing chemicals provided by the "Organization for Economic Cooperation and Development (OECD)".^{16,20,21}

In vivo anti-VL response

Male BALB/c mice (6–8 weeks, weight-matched) either didn't become infected or did become infected after intravenously receiving 2×10^7 *L. donovani* stationary phase promastigotes. Mice were ingested with vehicle control (DMA + Tween 80 + sterile water), uninfected control ($n = 6$), infected control ($n = 6$), and infected vehicle control ($n = 6$). Eugenol oleate (5, 10, and 30 mg/kg of B.W.) was given orally to three further infected groups ($n = 6$ per group at each time point) sequentially for three, five, or ten days. As the positive drug control, miltefosine was administered to $n = 6$ patients at a dose of 20 mg/kg B.W. every day for 5 days.²² All the mice were sacrificed on the 14th day following the first day of treatment. After Giemsa staining, tissue impressions were used to estimate the parasite burden in the spleen and liver. Leishman Donovan units (LDU), as mentioned in the prior report,¹⁵ were used to express results. To investigate NO production, arginase activity, T-cell proliferation, and Th1 and Th2 cytokine responses, splenocytes were separated from each group and restimulated with soluble leishmanial antigen (SLA).²⁰

Nitric oxide (NO) generation, Arginase activity and cytokines release assay

Nitrite accumulation in culture supernatants was estimated using the Griess method as described earlier.^{15,23} Arginase activity was assessed using a technique previously reported.²⁴ Following the manufacturer's instructions, cell-free supernatants were collected, and the release of cytokines into the culture supernatants was measured using sandwich ELISA kits (R & D Systems in Minneapolis, Minnesota, USA).^{15,20}

Isolation of RNA, mRNA expression study by real-time RT-PCR

Cells were collected in Trizol from different experimental sets, 1- μg of RNA from each set reverse transcribed (using Revert Aid MMuLV reverse transcriptase, Fermentas), amplified in real-time PCR (Eppendorf RealPlex master cyclor, using 2X SYBR premix Ex Taq II, TAKARA Bio), normalized against

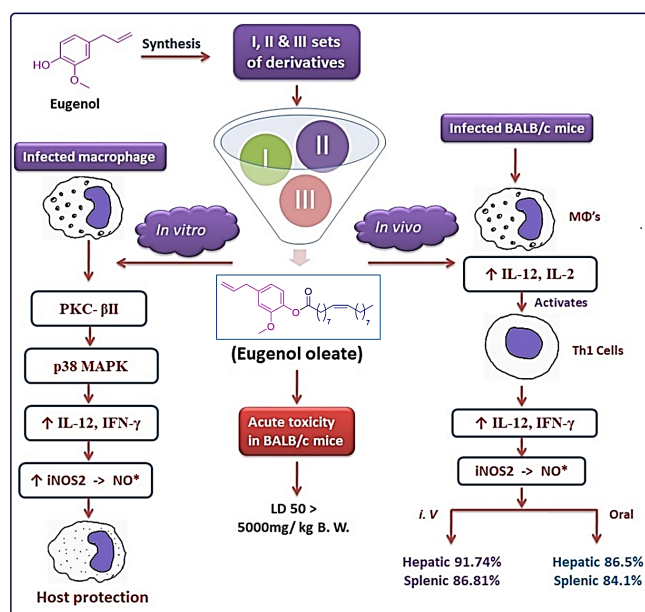


Figure 1: Schematic representation of the eugenol oleate mediated anti-leishmanial activity.

glyceraldehyde 3-phosphate dehydrogenase GAPDH levels, and quantified by 2-ddCT method.^{20,23-25}

Immunoblotting

Protein concentration was estimated from cell lysate using the Bradford method and proteins were transferred onto a polyvinylidene fluoride (PVDF) membrane by electrotransfer using Bio-Rad mini gel blotting apparatus. Primary antibody (1:1000 dilution) and secondary antibody (1:10,000 dilutions) were added followed by gentle rocking conditions for 2 hours at room temperature after each step unbound antibody was washed out. Finally, the membrane was washed with Tris-buffered saline (TBS) with 0.1% Tween-20 (TBST) followed by TBS buffer 3 times. The ECL substrate (Bio-Rad) was added to the membrane and kept for 5 minutes. The image was captured by using a Bio-rad gel documentation system connected to a computerized camera. The band intensities were estimated using Image J software (NIH), and each protein's intensities were represented as normalized values to their respective loading controls.²⁰

Statistical analysis

Data are calculated as mean values \pm standard deviation (SD). Using GraphPad Prism 6, a two-way analysis of variance (ANOVA) with Dunnett's multiple comparisons test was used to determine the significance of the variations in the mean values of the infected, treated, and untreated infected groups.

RESULTS AND DISCUSSION

Activity against promastigote and amastigotes

Using the resazurin assay, we assessed the IC₅₀s for each drug against *L. donovani* promastigotes. IC₅₀s appeared as 20.13

μM for Eugenol oleate. We found cellular disintegrations, a short length of flagella, and a shrinkage body in the structure of *L. donovani* parasites in SEM imaging. We studied the anti-amastigote activity in infected macrophages. The dose-response curve indicated the EC₅₀ of 4.25 μM , while the TI value of 82.24, which was improved vigorously than the AmpB and miltefosine, the standard drugs.¹⁵

PK and acute toxicity of Eugenol oleate on oral administration in BALB/c mice

The Plasma PK study showed that it reached its maximum concentration (C_{max}) within 0.5 h of administration with a half-life (T_{1/2}) and mean residence time (MRT last) of 1.91 h. An acute toxicity study in BALB/c mice revealed a 50% lethal dose (LD₅₀) of >5000 mg/kg B.W. and no sign of visible toxicity toward the internal organs.²⁰ Hence, Eugenol oleate has good pharmacokinetics and safety profiles for being considered as a potential oral drug candidate.

Oral Eugenol oleate on parasite burden in *L. donovani*-infected BALB/c mice

Oral anti-VL efficacy was studied in the BALB/c mice model. The most common manifestations of VL are enlargement of the spleen and liver due to increased parasite burden.³⁵ We found maximum hepatic (86.5%) and splenic (84.1%) parasite clearances in 10 days of the treatment regimen of Eugenol oleate (30 mg/ Kg B.W.) significantly ($p < 0.001$). The vehicle for the oral administration of Eugenol oleate didn't show any noteworthy effect on parasite clearance in hepatic and splenic tissues as desired. This was the first report on the oral efficacy of Eugenol oleate against experimental VL our group.²⁰

Eugenol oleate on host protective Th1 cytokine response and NO generation

L. donovani parasite differentially regulates Th 1 (IL-12, IFN- γ , and TNF- α) and Th2 (IL-10) cytokines profiles to establish the VL inside the host, where it enhances Th2 and abrogates Th1 cytokine responses.^{19,29} The study revealed that Th1 cytokines mRNA expression was significantly ($p < 0.01$) increased and decreased in the experimental group with Eugenol oleate compared to the untreated control. We further validated these by sandwiching ELISA.²⁰ Inducible nitric oxide synthase (iNOS)-2 and arginase-1 are the counter-regulating enzymes involved in the L-arginine metabolism. Eugenol oleate treatment induced the parasite clearance through the Th1-dependent production of anti-parasitic NO generation and the suppression of Th2 response and arginase activity²² This was in line with previous reports on intravenous and intraperitoneal administration of Eugenol emulsion.^{15,28} When compared to the untreated, uninfected control group, the 30 mg/kg B.W. of Eugenol oleate treatment group significantly ($p < 0.001$) elevated iNOS2 mRNA expression levels by 10.14 times. Comparing the treated with Eugenol oleate group to the untreated, uninfected group, NO production was likewise significantly ($p < 0.001$) 6.61 times higher in the

treated group. In contrast, infection control considerably ($p < 0.001$) boosted the level of arginase-1 mRNA expression by 7.59 times in comparison to the uninfected control. However, compared to the infection control without treatment, it was considerably ($p < 0.001$) reduced in the Eugenol oleate-treated group, and arginase activity likewise showed a similar tendency.²⁰ Hence, Eugenol oleate successfully proved its oral immunomodulatory potential.

CONCLUSION

Eugenol oleate showed the highest TI value and moderate EC50 value against amastigotes when compared to the two most available and recommended medications against VL, miltefosine, and amphotericin B. Further, this anti-VL lead, Eugenol oleate, proved its oral efficacy, which can be considered the 2nd oral drug candidate after miltefosine. Eugenol oleate prompted Th1-dependent nitrite generation, the signature of anti-VL immune response in experimental mice model (Figure 1). This study established the possible use of Eugenol oleate as an oral immunomodulatory treatment wing against VL.

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CONFLICT OF INTEREST

None to disclose.

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