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Oral combination of eugenol oleate and miltefosine induce immune response during experimental visceral leishmaniasis through nitric oxide generation with advanced cytokine demand

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ABSTRACT

Conventional therapy of visceral leishmaniasis (VL) remains challenging with the pitfall of toxicity, drug resistance, and expensive. Hence, urgent need for an alternative approach is essential. In this study, we evaluated the potential of combination therapy with eugenol oleate and miltefosine in *Leishmania donovani* infected macrophages and in the BALB/c mouse model. The interactions between eugenol oleate and miltefosine were found to be additive against promastigotes and amastigotes with $x\Sigma$FIC 1.13 and 0.68, respectively. Significantly ($p < 0.001$) decreased arginase activity, increased nitrite generation, improved pro-inflammatory cytokines, and phosphorylated p38MAPK were observed after combination therapy with eugenol oleate and miltefosine. >80% parasite clearance in splenic and hepatic tissue with concomitant nitrite generation, and anti-VL cytokines productions were observed after orally administered miltefosine (5 mg/kg body weight) and eugenol oleate (15 mg/kg body weight) in *L. donovani*-infected BALB/c mice. Altogether, this study suggested the possibility of an oral combination of miltefosine with eugenol oleate against visceral leishmaniasis.

1. Introduction

Clinical manifestation of leishmaniasis could be responsible for the cause of higher mortality and morbidity rates in the tropical and subtropical region worldwide, if remains untreated. Globally, severe form of visceral leishmaniasis (VL), caused by *Leishmania donovani,* was observed in South-eastern Asia, Africa and Indian subcontinent areas [1]. Besides that, several types of drug candidates are also available to treat leishmaniasis. Out of those candidates, miltefosine was recommended as an orally administered treatment for VL. Unfortunately, miltefosine treatment failure was reported in the Indian subtropical region, which recommended the liposomal amphotericin B as an alternative approach [2,3]. Moreover, the necessity of temperature-controlled cold chain assurance for restoration and expensive cost limited the use of liposomal amphotericin B [4,5]. Parasite resistance with miltefosine monotherapy was also identified experimentally [6]. Currently, the mutations in LdMT (*L. donovani* miltefosine transporter) and LdRos (potential non-catalytic β subunit of LdMT) were responsible for miltefosine resistance both *in vitro* and *in vivo* conditions [7]. However, it was reported that infection by *L. infantum* parasites became miltefosine resistance in Brazilian clinical sample [8]. More recently, other report demonstrated miltefosine resistant strains from India and Nepal due to its prolonged drug treatment exposure and association with various gene mutation [9]. Furthermore, miltefosine was not recommended for pregnant women [10]. However, further research on the efficacy of miltefosine is an ongoing way to confine its definite usage. Severe clinical complications and drawbacks of present therapy accentuate the urgent need to discover a new therapeutic regimen. Likewise, combination therapy might be the alternative approach to alleviate VL due to its better efficacy in minimal doses and lesser time of treatment. Being long half-life miltefosine could be a better potential regimen for combination therapy. In this context, oxabicyclic derivatives, 4-(7-hydroxy-4,4,8-trimethyl-3-oxabicyclo [3.3.1] nonan-2-yl) benzoate, showed synergic interaction in combination with miltefosine ($x\sum$FIC =

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0.088) against miltefosine responsive *L. donovani* strain [11]. Furthermore, an isobologram study with nelfinavir revealed the synergic (x∑FIC = 0.48) anti-leishmanial effect with miltefosine against *L. infantum in vitro* [12]. While, lopinavir showed additive anti-leishmanial interaction with miltefosine against *L. infantum in vitro* as well as *in vivo* [13]. Moreover, complications with recently acting drugs could overcome by combination therapy with other chemically synthesized compounds or natural compounds to control the disease.

Leishmania parasite intended to survive into host macrophages with the help of numerous mechanisms. VL was regulated by the release of TGF-β, IL-10 to induce Th-2 population and restricted host protective immune response. These Th-2 cytokines molecules dampened the release of IFN-γ, TNF-α, nitric oxide generation, and ROS which were involved in host protection [14]. Likewise, combination therapy inducing these anti-leishmanial immune response could be more beneficial.

Eugenol oleate is the chemically synthetic derivative of eugenol. Eugenol oleate showed better anti-leishmanial efficacy compared with other eugenol derivatives in terms of its therapeutic index and parasite clearance as evidenced by our group [15]. Recently, we reported that eugenol oleate exerted the anti-leishmanial immune responses with the involvement of p38 MAPK and iNOS2 [16]. It was also identified the oral efficacy of eugenol oleate beside the intravenous route of administration against experimental VL that attracts more attention on the anti-leishmanial potential of it. [15,16]. Indeed, we reported the synergic potential of eugenol oleate in combination with amphotericin B [17], in this study we intended to explain the oral efficacy of eugenol oleate combination with miltefosine against experimental visceral leishmaniasis. Besides that, we showed here the combinatorial association between these two with anti-leishmanial immune response *in vitro* and *in vivo*.

2. Materials and method

2.1. Ethics statement

The use of all laboratory mice was carried out with all the mandatory guidelines (CPCSEA Reg. No. 817/PO/ReRcBiBt/S/04/CPCSEA; Dated 13.12.2018) and was approved by the ethical committee of SASTRA Deemed to Be University (612/SASTRA/IAEC/RPP; dated 10.08.2019).

2.2. Animals and Leishmania parasites

Female BALB/c mice (6–7 weeks) were ingathered to continue this study. Animals were kept quarantined for 7 days before *L. donovani* infection and randomly allotted for 5 animals in each group for *in vivo* study. The *L. donovani* laboratory strain (MHOM/IN/AG/83) was routinely cultured in the BALB/c mouse model. To infect naive mice ex-vivo amastigotes were obtained from the splenocytes of heavily infected donor mice and allowed to transformed promastigotes. The promastigotes were cultured in M199 medium with 10% FBS and 1X PenStrep (Gibco) for further study. BALB/c mice were inoculated with the number of 2×10^7 parasites per mice with intravenous injection. The general condition of mice and body weight was maintained daily.

2.3. Synthesis of eugenol oleate

The synthesis procedure was described previously [15–17]. Briefly, oleic acid (1.8 mmol) was dissolved in dry DMF at 0 °C. To the well-stirred solution EDCI.HCl (1.65 mmol) and DMAP (0.15 mmol) were added in stirring conditions. Hereafter, eugenol (1.5 mmol) was added and kept for another 12 h at room temperature. After completion of the reaction, 10 ml distilled water was added to the reaction mixture and stirred for 30 min. The organic portion was then extracted by portioning with EtOAc (3 × 10 ml) and dried over anhydrous Na_2SO_4. The organic mass thus obtained by concentrating under reduced pressure was purified by chromatography to obtain the desired product (0.626 g, Yield 96%) as a colorless liquid with >99% purity by HPLC and LRMS [17].

2.4. Promastigote viability assay by resazurin assay

To determine the anti-leishmanial efficacy on parasites, stationary phase promastigotes seeded with 0.5×10^6 cells/ml with 10% FBS enriched M199 medium in 96 well plates and incubated for 48 h with different concentrations of drugs at 22 °C in a BOD incubator. Thereafter, resazurin was added at 50 μg/ml per well and incubated for another 24 h. After that fluorescence intensity was measured at 550 nm for excitation and 590 nm for emission using a multi-mode plate reader. Percentage (%) of viable parasites was calculated by using the mean of negative control as 100% survival of parasite [17,18].

2.5. In vitro anti-amastigotes killing assay within mouse peritoneal macrophages

Thioglycolate broth provoked peritoneal macrophages were isolated from BALB/c mice and used as described previously [17]. Macrophages were seeded in 2×10^5 cells/well with 10% FBS in RPMI medium in an 8 well chamber slide. After bringing them into proper differentiation and resting conditions, cells were infected with stationary phase promastigotes at 1:10 ratio for 4 h. After that non-phagocytozed parasites were washed by the use of 1X sterile PBS and were left for another 20 h incubation for further multiplication of *Leishmania* parasite inside the host cell. After that macrophages were replaced with fresh RPMI medium with the dose of eugenol oleate and miltefosine at the concentration of 0–10 μM each in a checkerboard type experimental setup. After 48 hr of incubation, the supernatant was removed and wells were washed with PBS and fixed with chilled methanol. The wells were Giemsa stained. The parasites/100 macrophages were counted with the help of an Olympus (BX43) microscope at 100× magnification and resolution in oil immersion for each group.

2.6. Isobologram construction and drug combination determination

Isobologram construction association curve was determined by using fractional inhibitory concentration (FIC) which is based on the following equation: IC_{50} of each drug in combination/IC_{50} of each drug alone. The sum of FIC (ΣFIC) calculated by FIC of eugenol oleate and FIC of miltefosine. The mean of the sum of FICs (xΣFIC) was calculated by the average of ΣFIC which compared with the reference value for combination and reported as synergistic xΣFIC = ≤0.5, additive ≥0.5–4 and antagonistic ≥4 [10,19].

2.7. Cytotoxicity assay

MTT assay was done to evaluate the toxicity of eugenol oleate and miltefosine in uninfected mouse peritoneal macrophages. BALB/C-derived macrophages were seeded with 2×10^5 cells per well in the 96 well plate. Eugenol-oleate and miltefosine were treated for 48 h. Hereafter MTT was added at 0.5 mg/ml and incubated for 3 h. Then the HCL-isopropanolic acid solution was added to each well to convert into MTT formazan product which was measured at 570 nm by using a microplate reader [15].

2.8. Morphological alteration by SEM imaging

SEM imaging was done to check the morphological variation in *L. donovani* promastigotes. Briefly, promastigotes (1×10^6) were seeded and incubated with 20 μM concentration of eugenol oleate with 1.25 μM of miltefosine in combination and individually for 24 h. After that promastigotes were washed two times with 1X PBS and fixed with 2.5% glutaraldehyde dissolved in 1X PBS at 22 °C for 2 h. Then suspensions

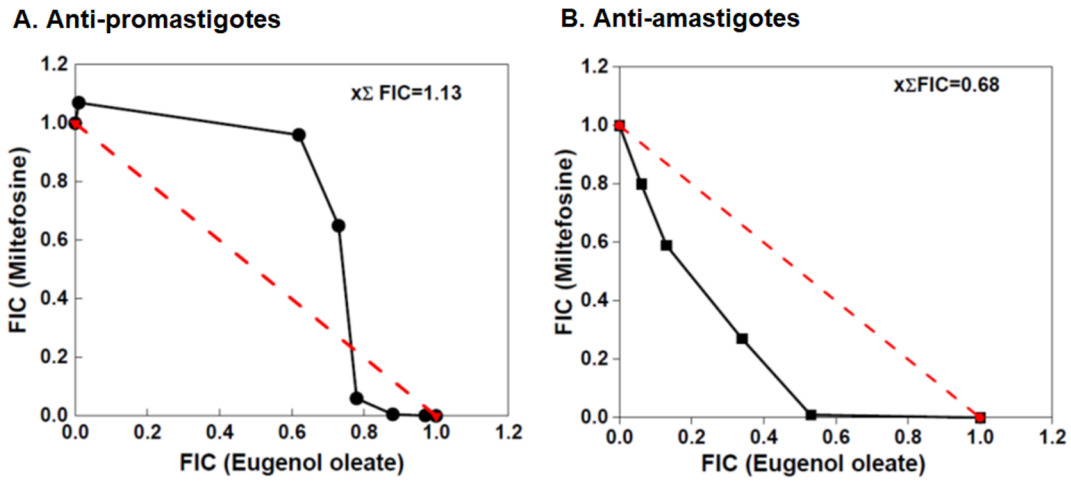

Fig. 1. *In vitro* drug association between eugenol oleate and miltefosine. Drug interaction was analyzed against *L. donovani* promastigotes (A) and intracellular amastigotes (B). Isobolograms indicate activity at the EC_{50} level. Plots were compared with the theoretical line ΣFICI = 1 at all ratio (dashed line) which represents no interaction between two compounds.

were washed to place on poly-L-lysine-coated coverslips and dehydrated with ethanol in ascending order. Dehydrated samples were dried by using a dryer to keep gold coating and observed under a Vega 3 scanning electron microscope [17,20].

2.9. In vivo efficacy in BALB/c mice

Male BALB/c mice (6 to 7 weeks) were infected through a tail vein with 2×10^7 stationary phase promastigotes per mouse. After 14 days, mice were treated orally with 5 mg/kg body weight (b.wt.) of miltefosine and 15 mg/kg b.wt. of eugenol oleate in combination and alone daily for 5 days. After 14 days of post completion of treatment, mice were sacrificed and the spleen and liver were collected aseptically to evaluate the parasitemia burden as Leishmania-Donovan units (LDU) by Giemsa staining.

To assess the post-treatment efficacy of the combination in infected mice, liver tissues were collected from five groups aseptically for the histopathological studies. After that tissues were placed for fixation in formaldehyde for the next 48 h and then gradually dehydrated with increasing concentrations of ethanol. Then tissues were embedded in paraffin block and sections were cut by using microtome to get thin tissue sections for Haematoxylin and Eosin (HE) stain. The stained slide was observed under a light microscope by using 10X and 40X objectives.

2.10. NO generation

Estimation of NO generation was done by using Griess reagent assay. The 100 µl of collected cell culture supernatants from *in vitro* and *in vivo* different set was mixed with 100 µl of Griess reagent and incubated at the dark condition for 25–30 min. The nitrite accumulation was measured at 548 nm in a plate reader.

2.11. Arginase-1 activity

The arginase-1 activity was measured in *L. donovani* infected macrophages [15]. After the treatment of combinatorial dose of eugenol oleate at 5 µM and miltefosine at 1.25 µM in infected macrophages, cells were lysed after 48 hr with 0.1% Triton X-100 with 25 mM Tris-HCl. Activation of arginase at 56 °C followed by arginine hydrolysis was carried out using 0.5 M *L*-arginine at 37 °C for 15–20 min. The urea concentration was measured at 540 nm after the addition of α-isonitrosopropiophenone followed by heating at 95 °C for 30 min. One unit of arginase activity was defined by the amount of enzyme catalyzed by the formation of 1 mol of urea/min.

2.12. Cytokine mRNA expression

To detect the mRNA profile of different cytokines *in vitro*, total RNA was isolated from experimental groups. Likewise, from *in vivo* set, total RNA was extracted from splenocytes of various groups of mice. 1 µg of total RNA was used as a template to synthesize cDNA. The specific forward and reverse primers were used to determine *iNOS2, IFN-γ, TNF-α, IL-10, IL-12,* and *arginase-1*. PCR cycling conditions were 5 min at 95 °C, denaturation followed by 35 cycles at 94 °C for 30 sec, annealing at 58 °C for 30 sec and extension at 72 °C for 1 min. PCR amplified gene products were run by 1.2% agarose gel electrophoresis and observed under UV light and analyzed in the Bio-Rad Gel documentation system. In real-time PCR, 2X SYBR premix (TAKARA Bio) was used to check the mRNA expression level of target genes which could be normalized against the levels of GAPDH and expressed as relative fold change compared with untreated control by the quantification of $2^{-\Delta\Delta CT}$ method [15,21].

2.13. Cytokine by ELISA

Cell-free supernatants were collected from peritoneal macrophages and splenocytes of various experimental groups to determine the cytokine level after 24 h of treatment by using an ELISA kit (R& D system, Minneapolis, MN, USA) following the manufacturer's instructions [15].

2.14. Immunoblot analysis

BALB/c derived peritoneal macrophages seeded in 6 well plates and infected with *L. donovani* stationary phase promastigotes and treated with a selective concentration of miltefosine and eugenol oleate. Then the adherent cell populations were collected from the plate and centrifuged at 2400 rpm for 15 min at 4 °C. The cell pellet was dissolved in RIPA buffer and the cell suspensions were sonicated by using a probe sonicator. The sample was centrifuged at 12,000 rpm for 15 min at 4 °C and protein in samples was estimated by the Bradford method. Then 50 µg of total protein were subjected to 10% SDS PAGE gel electrophoresis and transferred to PVDF membrane. The PVDF membrane was blocked by 5% milk protein in Tris-buffered saline with 0.1% Tween-20 (TBS-T) and immunoblotting was performed to evaluate the levels of protein expressions. The immunoreactive band was captured by Bio-Rad Gel Doc documentation system after visualized with ECL kit (Bio-Rad) and band intensities were analyzed by ImageJ software [16].

Table 1
IC_{50}s and FICs of eugenol oleate and miltefosine combinations against *L. donovani* promastigotes.

Form	Combination (μM)		IC_{50}(μM)		[a]FIC of		[b]ΣFIC
	Eugenol oleate	Miltefosine	Eugenol oleate	Miltefosine	Eugenol oleate	Miltefosine	
Promastigotes	160	0	23.71				
	80	0.3125	23.04	0.01	0.972	0.001	0.973
	40	0.625	21.01	0.04	0.886	0.005	0.891
	20	1.25	18.66	0.45	0.787	0.060	0.847
	10	2.5	17.34	4.86	0.731	0.651	1.382
	5	5	14.74	7.22	0.622	0.967	1.589
	2.5	10	0.16	8.04	0.006	1.078	1.084
	0	20		7.46			

[a] FIC, Fractional inhibitory concentration at the indicated IC_{50}.
[b] ΣFIC, Sum of the FICs.

Table 2
IC_{50}s and FICs of eugenol oleate and miltefosine combinations against *L. donovani* amastigotes in infected macrophages.

Form	Combination (μM)		IC_{50}(μM)		[a]FIC of		[b]ΣFIC
	Eugenol oleate	Miltefosine	Eugenol oleate	Miltefosine	Eugenol oleate	Miltefosine	
Amastigotes[c]	10	0	3.57				
	5	0.625	1.89	0.03	0.529	0.013	0.542
	2.5	1.25	1.22	0.62	0.342	0.273	0.615
	1.25	2.5	0.48	1.34	0.134	0.590	0.724
	0.625	5	0.24	1.83	0.067	0.806	0.873
	0	10		2.27			

[a] FIC, Fractional inhibitory concentration at the indicated IC_{50}.
[b] ΣFIC, Sum of the FICs.
[c] Intracellular *L. donovani*.

2.15. Statistical analysis

All the *in vitro* experiments were in triplicates. *In vivo* experiment was performed by using male BALB/c mice (n = 5 per group). Data represented as mean ± SD. IC_{50} calculation and isobologram construction were done by Origin Pro Software. Two-way ANOVA followed by

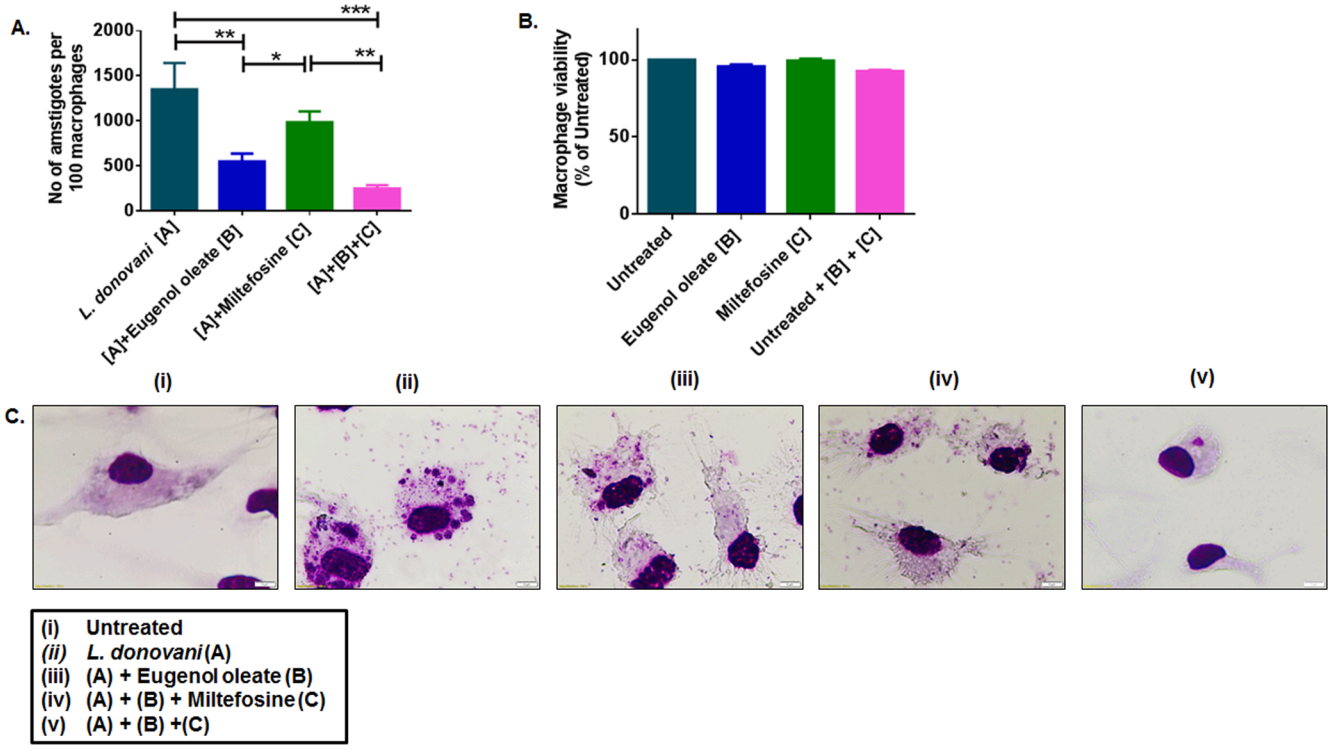

Fig. 2. Reduction of intracellular amastigotes inside murine macrophages. BALB/c peritoneal macrophages were seeded in 8 well chamber slides and then infected with stationary phase *L. donovani* promastigotes at 1:10 ratio. After 4hr wash with PBS to remove non-phagocytosed parasites and kept for 20 hr incubation. After 24 hr of post-infection well were replaced with fresh RPMI medium containing different concentrations of miltefosine and eugenol oleate. After 48 hr macrophages were fixed with chilled methanol and ready for Giemsa staining. Panel (A) shows amastigotes per 100 macrophages (B) cell viability measured by MTT assay and (C) representative image of macrophages. The data represent from at least three independent experiments (*and *** correspond to significant ($p < 0.05$ and $p < 0.001$).

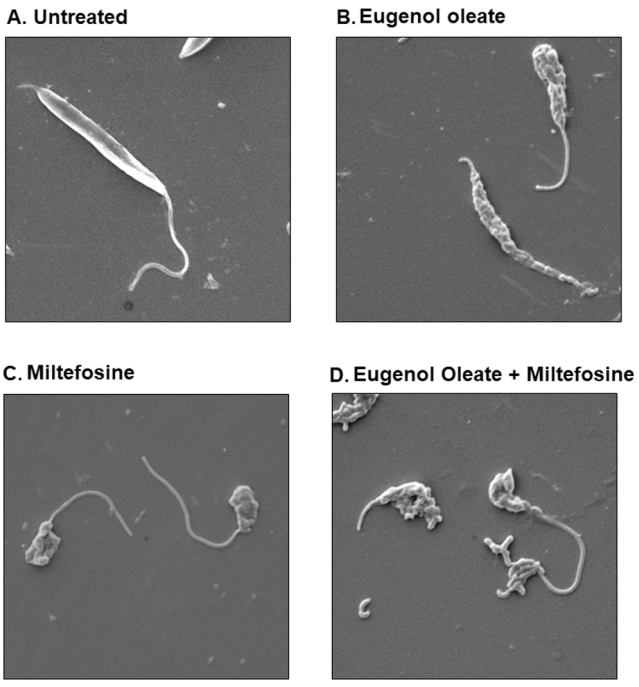

Fig. 3. SEM analysis of morphological changes in *L donovani* promastigotes. (A) Untreated control parasites (B) promastigotes treated with 20 μM of eugenol oleate, (C) with 1.25 μM of miltefosine and (D) treated with the combination of 20 μM of eugenol oleate with 1.25 μM of miltefosine.

Tukey's multiple comparison test was performed to note the significant difference among the experimental groups using GraphPad Prism 6.

3. Results

3.1. In vitro drug interactions of miltefosine and eugenol oleate against L. donovani promastigotes

We had assessed the EC_{50}s for each of the drugs against *L. donovani* promastigotes by using the resazurin assay. EC_{50}s were appeared as 23.71 μM and 7.46 μM for eugenol oleate and miltefosine, respectively. From this study, we had observed there was >85% killing at higher concentrations of these two drug regimens. The ΣFIC was calculated from the combination study by the isobologram method. The *x*ΣFIC was identified as 1.13, which indicated there was an additive interaction between these two compounds against *L. donovani* promastigotes (Fig. 1A, Table 1). Morphological changes of promastigotes were monitored by scanning electron microscopy (SEM). Fig. 3A showed untreated parasites, whereas, Fig. 3B-C represented the changes and cellular disintegrations in the structure of *L. donovani* parasites after the treatment of 20 μM of eugenol oleate and 1.25 μM of miltefosine, respectively. After given the combinatorial treatment, SEM analysis revealed the pronounced structural alterations in the *L. donovani* promastigotes with a short length of flagella and shrinkage body of the parasites (Fig. 3D).

3.2. Effect of eugenol oleate and miltefosine on cytotoxicity and parasite burden in L. donovani infected macrophages

To evaluate the anti-amastigote activity, we had used five concentrations of eugenol oleate (0–10 μM) and miltefosine (0–10 μM). In well accordance with anti-promastigotes results, the anti-amastigotes result of *in vitro* study showed additive interactions between eugenol oleate and miltefosine with *x*ΣFIC 0.68 (Fig. 1B, Table 2). We had selected 5 μM of eugenol oleate and 1.25 μM of miltefosine from the dose–response data for the further study. It was noteworthy that in that combination with 5 μM of eugenol oleate, 1.25 μM of miltefosine showed 81.69% killing of intracellular parasites within host macrophages (Fig. 2A–C). Interestingly, MTT assay revealed that the combination therapy with miltefosine and eugenol oleate didn't induce any significant toxicity at its selected doses (Fig. 2B).

3.3. In vitro alteration of nitric oxide generation, iNOS expression, and cytokine production in infected macrophages

Nitric oxide generation is an important mediator which helps to kill the intracellular parasite by boosting up the immune response [22]. In this present study, we had evaluated whether the proposed combinations of two drugs modulating NO generation or not. In infected macrophages, NO generation was increased significantly ($p < 0.01$) with 5.29 folds and 4.95 folds after the treatment of each drug alone than the infection control (3.522 ± 1.22 μM) respectively. While, the NO

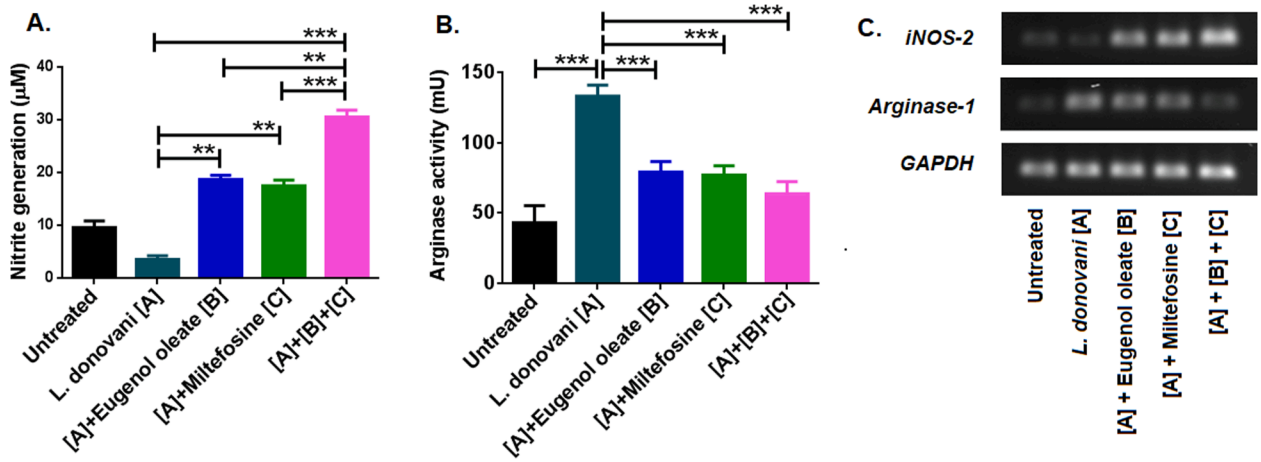

Fig. 4. Effect of the combination of eugenol oleate with miltefosine on NO generation and arginase activity in infected macrophages. (A) Data for nitrite generation expressed as mean ± SD from triplicate experiments, (B) Peritoneal macrophages were cultured, infected with *L. donovani* promastigotes followed by treatment with indicated concentrations of miltefosine and eugenol oleate as described earlier. After 48 h treatment, cell lysates were used for the arginase 1 activity by spectrophotometric method. The bar represents the mean ± S.D. of arginase 1 activity from three independent experiments. (C) After 6 h of treatment in infected macrophages, cell lysate was collected in Trizol, then RNA was extracted for cDNA preparation. mRNA expression was studied by conventional PCR using iNOS-2 and Arginase-1 specific primers. The data represent from at least three independent experiments.*, **, and *** correspond to the significant difference ($p < 0.05$, $p < 0.01$, and $p < 0.001$) compared among different indicated groups.

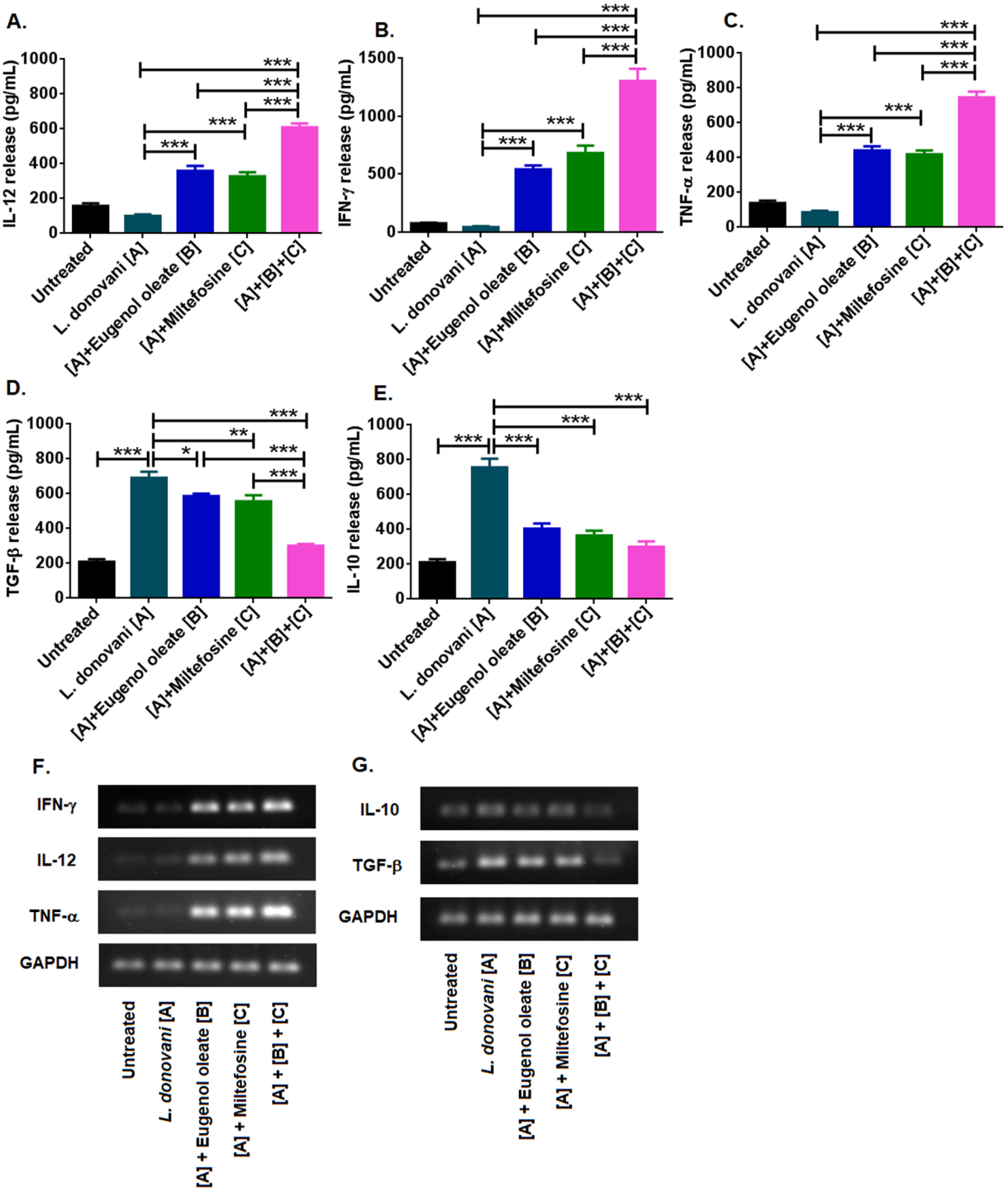

Fig. 5. Effect of combination treatment on pro and anti-inflammatory cytokines in *L. donovani* infected macrophages. Peritoneal macrophages were infected with 1:10 ration parasites and treated with 5 μM of eugenol oleate with 1.25 μM of miltefosine. (A–E) After 48 h of treatment cell-free supernatant was used to estimate the release of cytokines by Sandwich ELISA. *, **, and *** correspond to the significant difference ($p < 0.05$, $p < 0.01$, and $p < 0.001$) compared between different indicated groups. (F-G) a separate set of an experiment where treated cells were collected after 6hr of treatment and collected in TRIZOL and gene expressions were studied by RT-PCR with specific primers.

generation was increased 8.66 folds during the combinatorial treatment (Fig. 4A). Nitric oxide generation was dependent on the expression of inducible nitric oxide synthase (iNOS). Treatment with the combination dose showed a prompt increase in iNOS expression also in infected macrophages (Fig. 4C). In contrast, arginase activity and the arginase 1 expressions were decreased significantly ($p < 0.001$) after the treatment with the combination therapy (Fig. 4B–C). We also investigated the level of cytokines in infected macrophages after treatment with combination and alone of each drug by ELISA. The level of IL-12, TNF-α, and IFN-γ were significantly ($p < 0.001$) upregulated by 6.19, 8.92, and 30.37 folds respectively in combination-treated macrophages compared to infected control. Contrary to these observations, the level of IL-10 (2.53 folds) and TGF-β (2.31 folds) were decreased significantly ($p < 0.001$) compared with the untreated infected control group after the

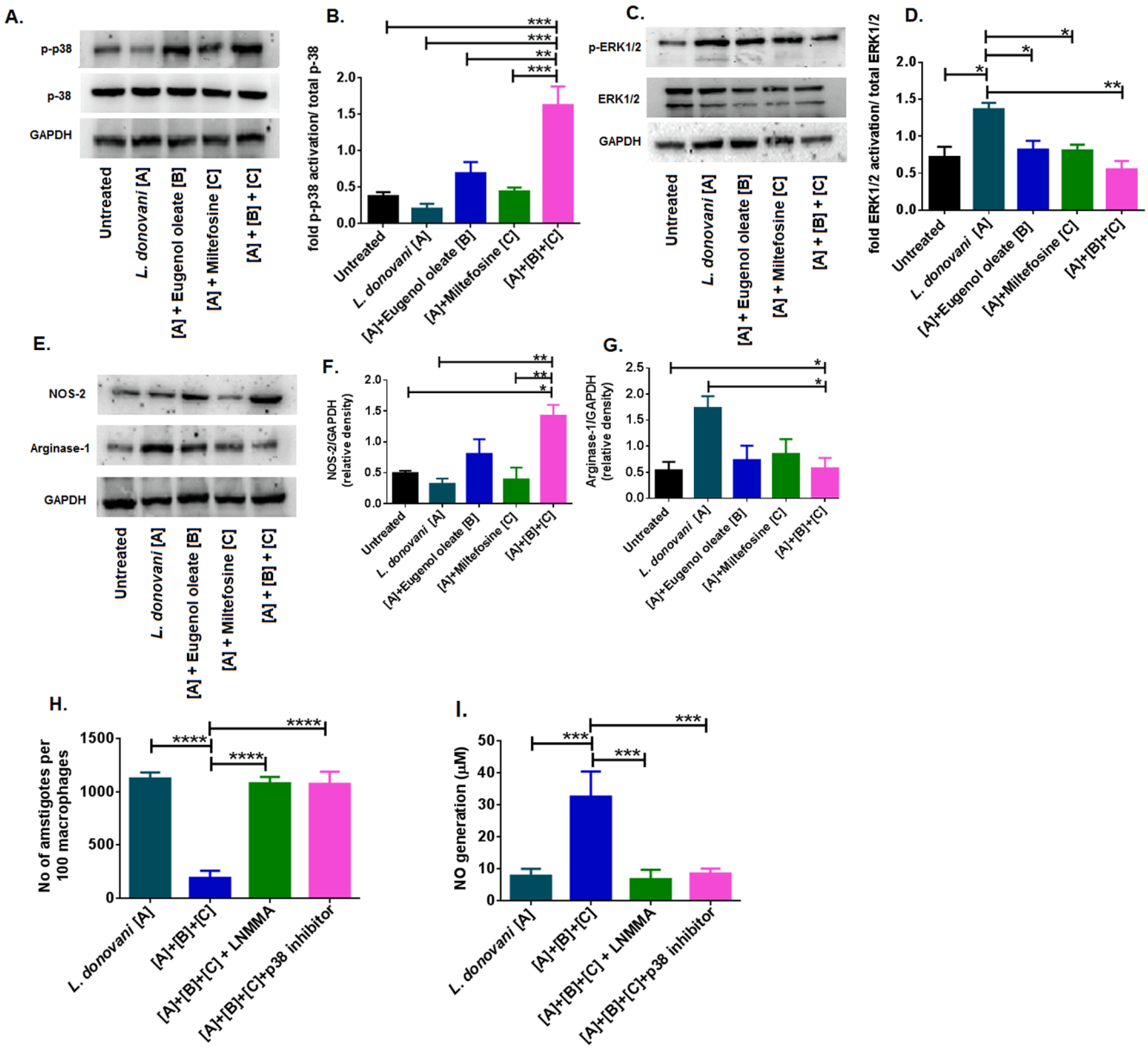

Fig. 6. Involvement of MAPKs in combination therapy eugenol oleate and miltefosine dependent NO generation and iNOS-2 expression in infected macrophages. Peritoneal macrophages were infected with *L. donovani* promastigotes for 4 h. After that un ingested parasites were removed by washing with RPMI without FBS and incubated for 20 h. Infected macrophages were treated with 5 μM of eugenol oleate and 1.25 μM of miltefosine alone or in combination for 30 min stimulation. Then the cells were lysed and subjected to western blotting with anti-pp38 MAPK (A), anti-pERK1/2 (C). After that western blotting for iNOS-2 and Arginase-1 expression. Densitometry analysis was performed by ImageJ 2.0 software (mean ± SD) (B, D, F, G). (H, I) *L. donovani* infected macrophages were pretreated with inhibitors [SB203580 (5 μg/ml) and LNMMA (0.4 mM)] for 1hr followed by treated with the combination of eugenol and miltefosine. After 48 h, cells were fixed with methanol, stained with Giemsa, and counted the parasite load per 100 macrophages at 100x objective, and the supernatant was used for NO generation by Griess assay. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$, ****$p < 0.0001$ significant differences compared with infected mice.

combinatorial treatment. Similarly, mRNA expressions of these cytokines were also altered in combinations of drug-treated macrophages (Fig. 5A–G).

3.4. Enhancement of phosphorylation of p38 MAPK along with the reduced phosphorylation of ERK1/2 by combinatorial treatment of eugenol oleate and miltefosine in infected macrophages

The MAPKs are the key regulators for IL-10 and IL-12 counter-regulation to maintain the anti-leishmanial cytokine balance. Earlier it was proved that *L. donovani* infection suppressed the level of IL-12 and other pro-inflammatory cytokine levels by the activation of IL-10 [23]. In this present study, we had examined the phosphorylation of p38 MAPK and ERK1/2 in infected macrophages after combinatorial treatment. Parasitization by *L. donovani* infection was resulted in pronounced induction of p-ERK1/2 in infected macrophages, whereas combinatorial treatment of eugenol oleate and miltefosine reduced the phosphorylation of ERK1/2. With the regard to p-38MAPK expression *L. donovani,* we observed that combinatorial treatment had shown a higher expression of p-p38 MAPK compared to infection control (Fig. 6A–D). The increased phosphorylation of p-38MAPK could play a key role to generate pro-inflammatory cytokines and NO generation by iNOS-2 activation [15,24,25]. Moreover, iNOS-2 expression was significantly increased by 4.39 fold than the infected set of macrophages after combinatorial treatment, while 2.49 fold and 1.21 fold increased after the treatment of eugenol oleate and miltefosine monotherapy alone,

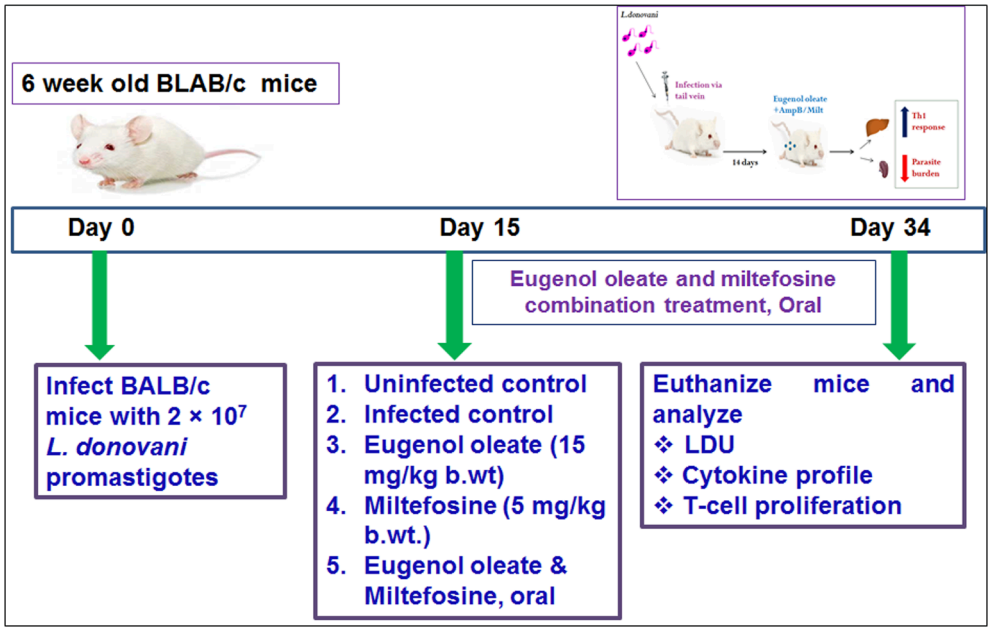

Fig. 7. Experimental design to evaluate the efficacy of eugenol oleate and miltefosine infected BALB/c mice.

respectively. Interestingly, immunoblot assay also confirmed that arginase-1 expression also significantly abrogated after the treatment of combination therapy with two-drug regimens in parasitized macrophages (Fig. 6E–G). To confirm whether the proposed combination enhanced NO release by the activation of p38MAPK, we studied the anti-leishmanial efficacy of combination with the pre-treatment of p-38 inhibitor (SB203580) and nitric oxide synthase inhibitor (L-NMMA; N^G-Monomethyl-L-arginine acetate) in *L. donovani* infected macrophages followed by the treatment with miltefosine and eugenol oleate. The anti-amastigote activity in terms of parasite load and NO generation were dampened significantly in the presence of these inhibitors (Fig. 6H–I). These results validated that the combination of miltefosine and eugenol oleate mediated the upregulation of p38MAPK accompanied by nitric oxide generation.

3.5. *In vivo anti-leishmanial activity of combination with miltefosine and eugenol oleate*

To study the *in vivo* efficacy of the combination therapy, *L. donovani*-infected BALB/c mice were treated with 5 mg/kg b.wt. of miltefosine and 15 mg/kg b.wt. of eugenol oleate alone or in combination for 5 consecutive days through oral route of administration (Fig. 7). The splenic and hepatic parasite load were calculated after the stamp smear method. There was a distinct parasite burden reduction of 88.76% and 80.6% in the liver and spleen respectively after combination treatment in infected BALB/c mice (Fig. 8A–B). Eugenol oleate alone (15 mg/kg b.wt.) showed 61.33% and 59.02%, miltefosine alone (5 mg/kg b.wt.) showed 57.49% and 43.34% of parasite reduction in the liver and spleen, respectively.

Additionally, histopathological analysis, specifically the formation of granuloma in the liver, was the one of major signs incorporated with *L. donovani* infection in mice. Hence, we checked the efficacy of eugenol oleate and miltefosine on hepatic granuloma in *L. donovani* infected BALB/c murine model. To implement this, liver tissues from different groups of mice were collected and prepared for histopathological examinations under the microscope. The results revealed that untreated sets of infected mice showed high levels of granuloma formation, in contrast, the combination therapy of eugenol oleate with miltefosine abled to clear granuloma formation in infected liver tissue. These results successfully demonstrated that the association between eugenol oleate and miltefosine significantly augmented the cure rate of *L. donovani* infection in BALB/c mice (Fig. 8C).

3.6. *Efficacy of combination therapy in T-cell proliferation with IL-2 release and Th-1/Th-2 cytokine balance*

Disease progression after the *L. donovani* infection was correlated with the impaired T-cell proliferation and pro-inflammatory cytokine released, while, healing desired higher T-cell proliferation and boost up host immune response [26]. Therefore, T cell proliferation was investigated after the combination therapy with eugenol oleate and miltefosine. As observed, splenocytes from *L. donovani* infected mice were incapable to induce T-cell proliferation in presence of 10 μg/ml of SLA (Soluble Leishman promastigotes antigen). Interestingly, combination treatments with miltefosine (5 mg/kg b.wt.) and eugenol oleate (15 mg/kg b.wt.) increased significantly the T cell proliferation by 19.51 folds compared to the infection control group. Miltefosine and eugenol oleate alone showed moderate T cell proliferation of 14.70 folds and 15.20 folds, respectively (Fig. 9I). *L. donovani* tends towards the form of anti-inflammatory Th-2 cytokine to survive in the host body, whereas, host immune response against *L. donovani* demands higher levels of Th-1 cytokine [27]. Hence, the cytokine profile was assessed by ELISA and mRNA expression from splenocytes of various groups. ELISA data resulted that the level of IL-12 and IFN-γ release were enriched in the combination-treated group by 11.34 folds and 15.14 folds, respectively than the infected group. Whereas, IL-10 and TGF-β were induced significantly to 5.44 folds and 3.97 folds in the infected group than the uninfected control group. These, IL-10 and TGF-β, were significantly diminished after the combination therapy. These cytokines were modulated by following the similar trend in the mRNA expression study also (Fig. 9A–H). Moreover, IL-2 release were also increased by 4.92 folds after combination treatment compared to infected group (Fig. 9J). All the data from *in vivo* experiments confirmed that the combinations of two drugs abled to induce T-cell proliferation with Th-1 cytokines release to combat parasite burden in infected BALB/c mice (Fig. 9A–J).

3.7. *Nitric oxide release and iNOS expression in infected BALB/c mice*

Nitric oxide is a crucial factor to reduce *L. donovani* infection [16]. Host protective immune response depends on macrophage-mediated NO

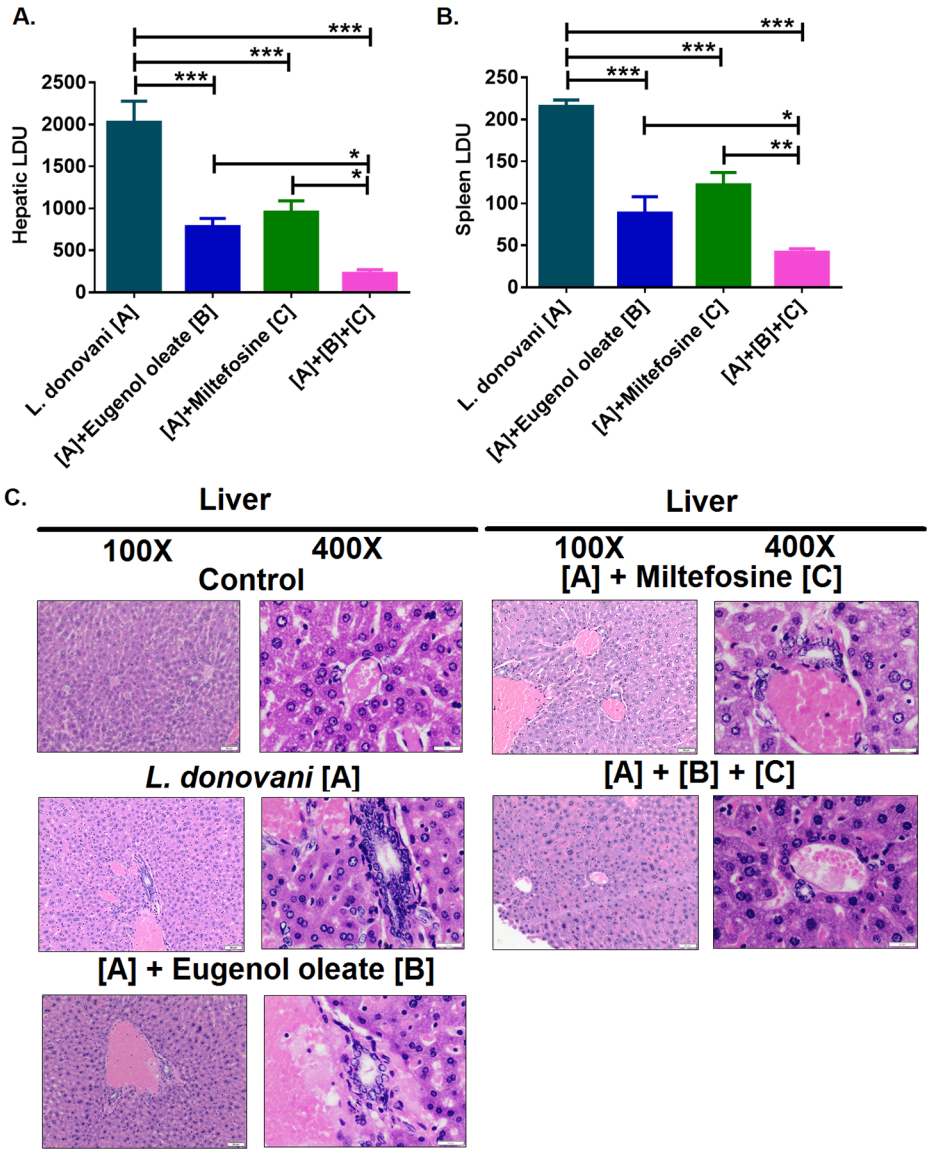

Fig. 8. Effect of eugenol oleate and miltefosine in combination and monotherapy on parasite burden in *L. donovani* infected BALB/c mice model. Mice had infected with 2×10^7 parasites per mice and after 15 days of post-infection, 15 mg/kg b.wt of eugenol oleate and 5 mg/kg b.wt of miltefosine treatment in monotherapy and combination were given to mice through oral administration route of injection for 5 consecutive days. After 14 days of post-treatment mice were sacrificed and liver and spleen were collected aseptically to investigate hepatic and splenic LDU from Giemsa tissue imprint. Results were expressed as mean ± S.E.M. of n = 5 per group. *p < 0.05; **p < 0.01; ***p < 0.001, significant differences compared with infected mice. (C) histopathological analysis of liver sections. Liver sections were stained by HE staining described elaborately in materials and method part.

generation to kill the intracellular parasite trapped into the phagolysosomes. Therefore, the mRNA level of iNOS had shown maximum after the combination treatment with eugenol oleate and miltefosine. This was fairly more than the infection group. Similarly, splenocytes from the combinatorial treated group induced a NO generation maximum of 34.99 ± 5.25 μM than the infected group. These demonstrated that the combination of eugenol oleate and miltefosine were abled to increase NO generation by iNOS expression in splenocytes (Fig. 10A–B).

4. Discussion

The development of resistance and toxicity of the existing drugs have been persisted with major drawbacks for conventional therapies of leishmaniasis [10], which can be overcome with combination therapy as the best alternative strategy [28]. Moreover, miltefosine resistance cases were initially described in clinical reports of HIV co-infected patients in France, as well as, in Indian patients [5,29]. Previously, after treatment with miltefosine, relapse rate raised at 20% in Nepal [30]. Although, being an orally-administered drug and having high cure rate, miltefosine was granted as a new hope to treat leishmaniasis in a highly endemic area.

Eugenol oleate, the synthetic derivative of eugenol, proved its oral anti-leishmanial immunotherapeutic potential earlier [16]. Progressive research with eugenol oleate also proved the synergic potential with amphotericin B [17]. Various studies also suggested that the anti-leishmanial efficacy of different conventional, as well as, new drugs were improved in combination with miltefosine, the only oral drug available in the market from the past two decades [31,32].

The present study aimed to appraise the anti-leishmanial efficacy of eugenol oleate and miltefosine combination using *in vitro* and *in vivo* murine model of experimental visceral leishmaniasis. At first, we determined the nature of the interaction between these two drug regimens *in vitro*. Isobologram indicated the association between these two compounds ($x\Sigma$FIC 1.13 and $x\Sigma$FIC 0.68) and showed additive interaction. Similarly, other studies with tamoxifen and miltefosine showed an additive effect against *L. amazonensis* promastigotes and amastigotes ($x\Sigma$FIC 1.32 and 0.63) [33]. Likewise, the combination of apigenin and miltefosine exhibited additive interaction ($x\sum$FIC = 1.61) against *L. amazonensis* intracellular amastigotes *in vitro* [34]. The result obtained from the present *in vitro* study well documented that the combination between eugenol oleate and miltefosine was not synergic against *L. donovani*, but, eugenol oleate could not pulverize the efficacy of miltefosine. Besides that, our results from the dose-dependent study also

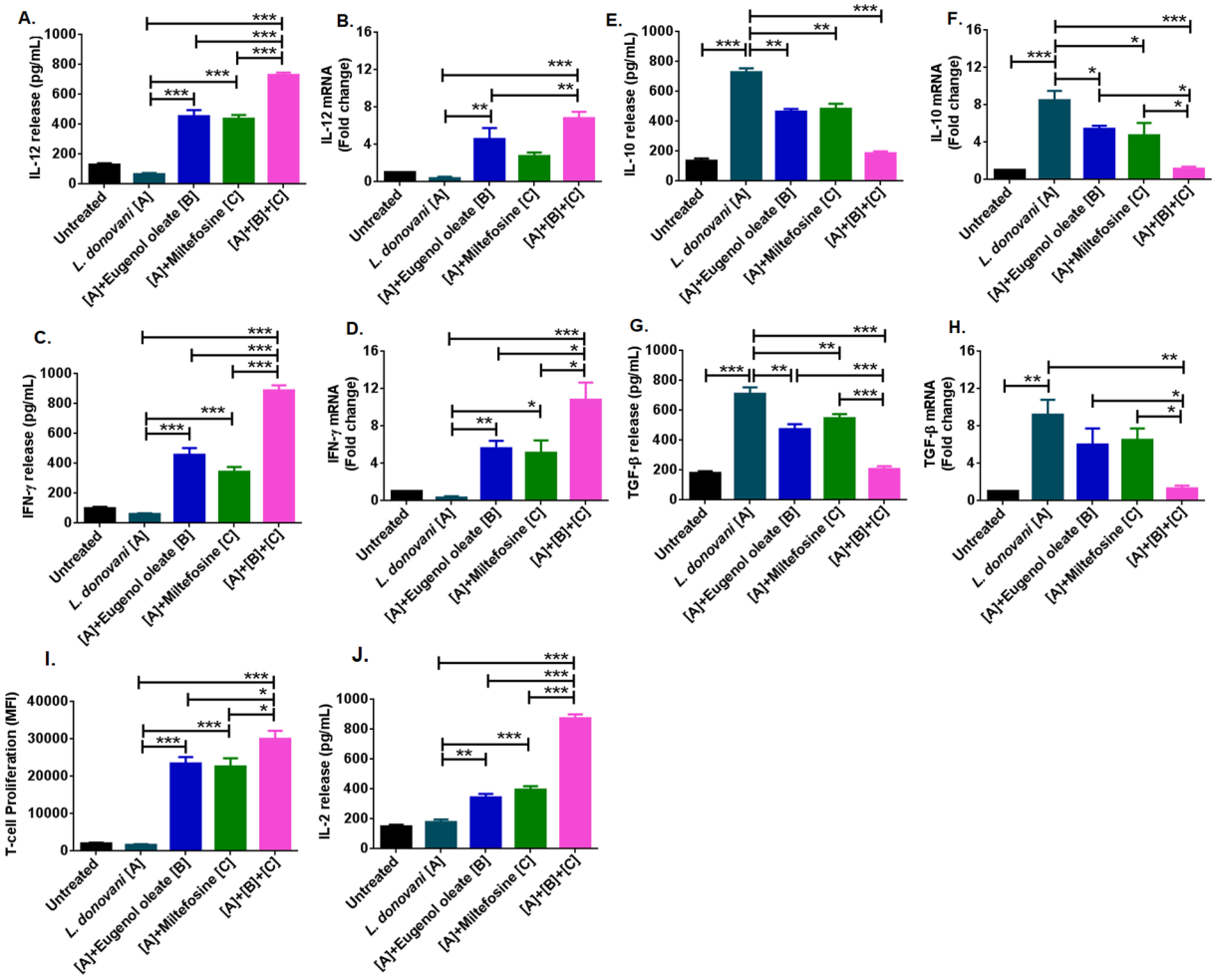

Fig. 9. Effect of combination treatment on Th-1 and Th-2 cytokine release in infected BALB/c mice. Isolated splenocytes from different groups were isolated and stimulated with 10 μg/ml of SLA for 72 h at 37 °C at 5% CO_2. (A–H) Supernatants from cultured splenocytes were evaluated for determination of IFN-γ, IL-12, TGF-β, and IL-10 levels by sandwich ELISA and PCR, (I) T cell proliferation was assessed by Resazurin assay and (J) IL-2 release by ELISA assay.

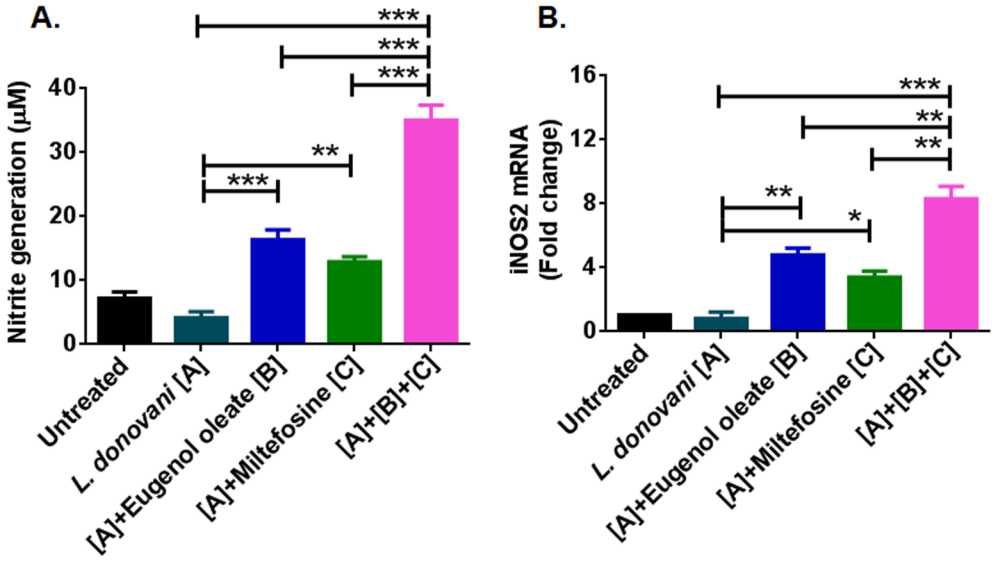

Fig. 10. Effect of eugenol oleate with miltefosine on nitrite generation of *iNOS2* expression in splenocytes of *L. donovani* infected BALB/c mice. (A) Isolated splenocytes from indicated groups were seeded aseptically (2×10^6) and stimulated with SLA (10 μg/ml) for 48 h after that nitrite generation was estimated from the cell-free supernatant using Griess reagent. Data represented here as mean ± S.E.M. of n = 5 mice per group. (B) iNOS2 mRNA expression was determined by quantitative real-time PCR from separate sets of splenocytes collected in Trizol as described previously. Data represented here as mean ± S.E.M. of n = 3 per group. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, significant differences between the indicated groups.

appraised a significant ($p < 0.001$) reduction in intracellular parasite burden at a selected concentration of 1.25 μM of miltefosine and 5 μM of eugenol oleate. Morphological changes also confirmed the combinatorial effect of those compounds on direct parasite killing (Fig. 3).

Macrophages are the hostile environment to survive for parasite

inside its. However, *in vitro* study with miltefosine and eugenol oleate abled to induce the significant ($p < 0.01$) level of NO (30.51 ± 2.28 μM) than the infected and monotherapy by iNOS expression facilitating towards parasite clearance (Fig. 4).

As expected, the levels of pro-inflammatory cytokines were upregulated after the combination treatment (Fig. 5). Infection was established due to the differential production of pro and anti-inflammatory cytokines regulated by the MAPK signaling pathway [35]. To explore the pathway behind this combinatorial treatment, MAPK signaling was checked in infected macrophages. The combinatorial effect of eugenol oleate (5 μM) and miltefosine (1.25 μM) in *L. donovani*-infected macrophages was accompanied by the increase of p38MAPK phosphorylation (Fig. 6) to induce IL-12 release and decrease in IL-10 [16,35]. Moreover, the inhibitor response study confirmed that the swift NO generation and declined parasite load were introverted in presence of an inhibitor of NO (L-NMMA) and p38 inhibitor (SB203580) even after combination therapy with eugenol oleate and miltefosine (Fig. 6H–I). Hence, results obtained from the *in vitro* study established that the combination of eugenol oleate and miltefosine exerted its anti-leishmanial effect against intracellular parasites by inducing pro-inflammatory cytokines through enhanced phosphorylation of p38MAPK (Fig. 6).

Even in the lack of synergic interaction between eugenol oleate and miltefosine observed *in vitro* study, that combination presented various advantages such as low doses, short period, and specifically oral route of administration (Fig. 7). *In vivo* study also confirmed that there were 88.76% and 80.6% of hepatic and splenic parasite clearance respectively after the treatment of 15 mg/kg b.wt. of eugenol oleate and 5 mg/kg b.wt. of miltefosine in infected BALB/c mice model (Fig. 8). Not only that, the combinatorial treatment increased T-cell proliferation by IL-2. Interestingly, interfered NO generations and iNOS expressions in splenocytes were amended after the combination therapy. The treatment of eugenol oleate in combination with miltefosine altered the shifting from Th-2 intra-macrophage infection state to Th-1 disease resolving protective state by inducing the levels of TNF-α, IFN-γ, and IL-12, while, diminishing the levels of IL-10 and TGF-β in the host cell (Fig. 9). Recently, it was reported that curcumin nanoparticles (CNP) have synergistic activity with miltefosine against promastigotes and amastigotes. Eventually, CNP showed 85% parasite reduction *in vivo* after treatment of CNP (25 mg/kg b.wt.) and miltefosine (10 mg/kg b.wt.) [36]. It was also reported that the combination of pyrazolopyridine derivative at 25 mg/kg b.wt. with 5 mg/kg b.wt. of miltefosine inhibited >97% parasite burden in *L. donovani* infected BALB/c mice [27].

AmBisome was not recommended as monotherapy against *L. donovani* in the Indian subcontinent region because of its high doses and cost-effectiveness. It was also become ineffective against African *L. donovani* infection and the combination of miltefosine and AmBisome was already considered for the alternative approach [37]. It is also noteworthy that oral drug administration is the simplest method by which patient alone can take it without help from trained medical personal. Moreover, oral drugs are easily absorbed inside oral mucosa and enter into systemic circulation bypassing the gastrointestinal tract and first-pass metabolism in the liver [38]. Therefore, the studied oral combination of eugenol oleate and miltefosine may have great impact in future.

Collectively, the *in vitro* and *in vivo* data validated the studied oral combination of eugenol oleate and miltefosine that abled to reduce drastic parasitic burden through NO generation and polarized macrophage toward disease resolving state by phosphorylation of p38MAPK. The mentioned combination therapy was comprising herein as an immunomodulator (eugenol oleate) with anti-leishmanial (miltefosine) presenting better efficacy against *L. donovani* to improve the immune response.

CRediT authorship contribution statement

Amrita Kar: Conceptualization, Methodology, Validation, Investigation, Formal analysis, Data curation, Writing - original draft, Writing - review & editing. **Mamilla R. Charan Raja:** Methodology, Investigation. **Adithyan Jayaraman:** Methodology, Validation, Investigation, Formal analysis, Writing - review & editing. **Sujatha Srinivasan:** Methodology, Investigation. **Joy Debnath:** Conceptualization, Methodology, Investigation, Formal analysis, Data curation, Resources. **Santanu Kar Mahapatra:** Conceptualization, Methodology, Formal analysis, Resources, Writing - original draft, Writing - review & editing, Visualization, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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