

# An Evaluation of Antileishmanial and Immunomodulatory Effects of Nitazoxanide and Pentoxifylline on Leishmania Tropica in Human U937 Macrophage

Khawla Adnan AlKhalidy

khawlaa.alkhalidy@student.uokufa.edu.iq

University of Kufa College of Medicine <https://orcid.org/0009-0000-4161-906X>

Sarmad Nory Gany

University of Kufa College of Medicine

---

## Research Article

**Keywords:** Nitazoxanide, Pentoxifylline, Leishmania tropica, Combination index, iNOS, Nitrite

**Posted Date:** October 16th, 2026

**DOI:** <https://doi.org/10.21203/rs.3.rs-10940127/v1>

**License:**   This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

---

# **An Evaluation of Antileishmanial and Immunomodulatory Effects of Nitazoxanide and Pentoxifylline on Leishmania Tropica in Human U937 Macrophage**

*Khawla Adnan Baqir*

*Department of Pharmacology and Therapeutics, Faculty of Medicine, University of Kufa, Kufa, Najaf*

*khawlaa.alkhalidy@student.uokufa.edu.iq*

*ORCID: 0009-0000-4161-906X*

*Prof. Sarmad Nory Gany, PhD*

*Department of Pharmacology and Therapeutics, Faculty of Medicine, University of Kufa, Kufa, Najaf*

## **Abstract**

Leishmania tropica parasite can cause cutaneous leishmaniasis, a disease whose current treatments, which depend on monotherapy, are unsatisfactory because of their high cost, resistance, and toxicity. Therefore, this study aims to suggest a better treatment that is based on a combination therapy using nitazoxanide (NTZ) and pentoxifylline (PTX) in human U937 macrophage by identifying the best doses of both drugs alone and in non-constant ratio combinations under two conditions before and after in vitro exposure of macrophages to L. tropica.

To achieve its aims, this study employs MTT assay to measure the viability effects of NTZ and PTX. Then, combination index (CI) and dose reduction index (DRI) are calculated by Chou Talalay method. inducible Nitric Oxide Synthase iNOS Gene expression is measured by qPCR and its weapon Nitrite is measured by Griess method.

The results showed that in non-constant ratio combination of 12.5 µg/ml NTZ and 250 µg/ml PTX, the viability percentage of L. tropica AMO significantly decreased (p value <0.0001) as compared with the infected control group. The calculated CI is 0.11196 which indicates a strong synergism. Optimal synergy is at the fa of 0.7203 which corresponds to 12.5 µg/ml NTZ combination dose and the DRI of NTZ is 10.9018. Moreover, the post-phagocytosis study demonstrated that the optimal drug combination successfully triggers a massive upregulation of (iNOS) gene expression and nitrite (NO<sub>2</sub>-) release by macrophages exposed to L. tropica.

Keywords: Nitazoxanide; Pentoxifylline; Leishmania tropica; Combination index; iNOS; Nitrite

## **Introduction**

More than one billion people in the world are infected by leishmaniasis; yet, this tropical disease is rarely studied (Cosma et al., 2024). As a complex parasitic disease, it results from intracellular protozoan parasites of genus leishmania humans receive when bitten by infected female phlebotomine sandflies (Torres-Guerrero et al., 2017). Such flies are infected by feeding on the blood of a previously infected mammalian host (Irshad & Khalid, 2022).

According to the 2022 WHO report, Algeria, Afghanistan, Brazil, Colombia, Iraq, Iran, Peru, and Syria reported thousands of leishmaniasis cases (Ruiz-Postigo et al., 2023) (mondiale de la Santé & Organization, 2023). In addition, Al Zadawi states that two kinds of cutaneous leishmania (CL) were

found in Iraq: zoonotic caused by *Leishmania major* and anthroponotic caused by *Leishmania tropica* (Al Zadawi, 2022). Currently, leishmaniasis affect about ninety countries where about thirty thousand cases of visceral leishmaniasis are reported, in addition to one million cases of cutaneous leishmaniasis (WHO, 2025) (Nunes et al., 2026). In 2026, leishmaniasis is still a significant global health problem because of the high mortality rate resulting from untreated cases (Bhattacharyya et al., 2026).

Iraqi MOH has reported a high incidence of CL (Al Zadawi, 2022) because of the aetiological agents responsible for cutaneous leishmaniasis which are wars, widespread violence, unlawful housing, deficiency in municipal services, Internal displacement of population, heaps of garbage, and terrorism, which have increased in Iraq after 2003.

Macrophages are vital, within the immune system, in fighting against *Leishmania* parasites, acting as host cells as well as vigorous contributors to the immune response. The status of macrophage activation determines the outcome of *Leishmania* infection, as they play a dual role during leishmaniasis (Keyhani et al., 2025). In their life cycle, *Leishmania* parasites are found in two developmental stages: "flagellar promastigotes (PRO), which replicate in the midgut of the sand fly and intracellular amastigotes (AMO), which reside inside the phagolysosomes of macrophages in the mammalian host" (Abadías-Granado et al., 2021).

After an infection, protection from leishmaniasis should involve recruiting and activating inflammatory monocytes whose role is to restrict parasite proliferation via producing reactive oxygen *species* and inducible nitric oxide synthase (iNOS) (Loría-Cervera & Andrade-Narvaez, 2020). Nitrite ( $\text{NO}_2^-$ ) constitutes a stable byproduct formed through the interaction of nitric oxide (NO) with oxygen and water; consequently, its accumulation in the culture medium serves as a reliable proxy for total NO synthesis (Maksouri et al., 2017). Additionally, elucidating the dynamics of macrophage-parasite interactions is crucial in deepening our comprehension of the underlying pathophysiology, ultimately paving the way for novel therapeutic interventions (Abbas & Ali, 2024). Current treatments exhibit considerable limitations, among which are long treatment durations, invasive administration, emergence of resistance and the high cost. Therefore, new strategies in leishmaniasis treatment development have been applied: drug repositioning and immunomodulation. While the former has gained approval to most recent therapeutic compounds, the latter has enhanced treatment efficacy and lower relapse when used in conjunction with standard chemotherapies (Majoor et al., 2025). Sodium stibogluconate and meglumine antimoniate, among other pentavalent antimonial compounds, act as the first-line pharmacological agents for leishmaniasis. Nonetheless, specialists have raised serious concerns about possible resistance, high treatment costs, and toxicity (Aksoy & Kilimcioğlu, 2025). Presently, vaccines like *Leishmania* fractionated vaccine, recombinant vaccine, DNA vaccine, antigen-cocktail vaccine and chimeric vaccine receive due attention in research and development trajectory exhibiting promising therapeutic applications (Choudhari et al., 2026). For the treatment of leishmaniasis, nowadays, anti-parasitic drugs are considered novel therapeutic candidates (e. g. increased production of reactive oxygen species and inhibit redox enzyme) (Bashir et al., 2023).

Nitazoxanide [2-(5-nitrothiazol-2-ylcarbamoyl) phenyl acetate] is a broad-spectrum anti-parasitic and antiviral agent belonging to the thiazolide class of nitroheterocyclic compounds. Demonstrating clinical efficacy against a diverse array of protozoan, helminthic, and viral infections, NTZ exerts its parasitocidal effects by inhibiting pyruvate ferredoxin oxidoreductase (PFOR). This inhibition fundamentally disrupts ATP synthesis, ultimately leading to parasite death (Al-Kuraishy et al., 2022) and (Khalid et al., 2022). Because ATP generation inherently relies on glycolysis—a process catalyzed by pyruvate kinase—the disruption of this cellular energy pathway positions NTZ as a highly promising candidate for inhibiting *Leishmania* parasites (Gurgen et al., 2011). Furthermore, NTZ possesses the potential to circumvent drug resistance pathways driven by enzymatic mutations. It achieves this by impeding the metabolic cascade at its initial stage, specifically preventing pyruvate from binding to the thiamine pyrophosphate cofactor, rather than competing directly with downstream cellular substrates (Khalid et al., 2022).

Pentoxifylline, a methylxanthine derivative traditionally indicated for vascular and chronic inflammatory conditions, acts as a potent inhibitor of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ). By downregulating hyperactive immune responses, PTX effectively mitigates localized tissue inflammation. In the context of leishmaniasis, integrating PTX as an adjunct to standard antimonial therapy significantly enhances overall cure rates for both mucosal and cutaneous manifestations (Brito et al., 2014). Additionally, this adjunctive approach accelerates lesion healing and markedly decreases the incidence of therapeutic failure (Cincura et al., 2022).

Given these distinct mechanistic advantages, combinatorial therapies are increasingly recognized as essential, customizable frameworks for advanced disease management (Choudhari et al., 2026). Deploying multidrug regimens serves as a robust strategy to overcome the persistent limitations of current anti-leishmanial monotherapies. By integrating agents that possess complementary or orthogonal mechanisms of action, this approach aims to maximize therapeutic efficacy and drive synergistic clinical outcomes. However, the successful translation of these multidrug protocols into standard clinical practice necessitates comprehensive, large-scale trials to precisely calibrate dosages and validate clinical efficacy (Majoor et al., 2025).

Consequently, the current study aims to estimate the cytotoxicity based on viability upon exposure of U937-macrophages cell line to *Leishmania tropica* and serial concentrations of NTZ and PTX alone and in non-constant ratio combinations, which are used under two conditions: before and after exposure to *Leishmania tropica*. It also investigates the effects of NTZ and PTX treatment on gene expression of iNOS and Nitrite levels.

## **Materials and Methods**

### **L. tropica isolate**

This study utilized Iraqi strain of *L. tropica* (MHOM/IQ/2011/MRU19) which was obtained from the parasitology laboratory for postgraduate studies at the Department of Biology, College of Science, University of Baghdad. The strain was then cultured in a biphasic medium (Nove-MacNeal-Nicolle).

### **U937 cell-line**

The promyelocytic human myeloid leukaemia cell line (U937) was used after it was purchased from Rawafid-Aleloom company for research services, Hillah-Iraq. The U937 monocyte was supplied by the National Cell Bank of Iran (Pasteur Institute of Iran NCBI C130). The cell line was cultivated using Roswell Park Memorial Institute (RPMI-1640) culture medium (Gibco, UK).

### **Parasite culture**

Promastigote forms of *Leishmania tropica* were cultured in RPMI 1640 liquid media in cell culture flasks with a filter cap of twenty-five cm<sup>2</sup> (Falcon/USA) at a pH of 7.4, supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco, UK). In addition, 100 IU/mL of penicillin and 100 mg/mL of streptomycin (Gibco, UK) was added to media. The culture was maintained at a temperature of 26°C (Hassan & Ali, 2020). The cultures were regularly subcultured and observed under a light microscope to monitor their growth and viability (Neamah et al., 2022).

### **U937 Cell line Culture**

U937 is the frequent monocytic cell line used as macrophage precursors due to their ability to echo the process of macrophage differentiation and because they are similar to primary monocytes and macrophages in morphology and biological responses. U937 cells are derived from histiocytic lymphoma (male patient, 37 years old) (Nascimento et al., 2022). The U937 cell line was cultured in RPMI1640 media supplemented with 10% heat-inactivated FBS filtered using a double sterile syringe filter with

pore sizes of 0.22 micron supplemented with 1% penicillin/streptomycin. Cell cultures were maintained at 37°C under a humidified atmosphere containing 5% CO<sub>2</sub>.

Treatment with phorbol 12-myristate 13-acetate (PMA) (Invevo Genes, USA) triggers the differentiation of monocytic cells into unpolarized macrophages (M0), which can then be selectively polarized toward either pro-inflammatory (M1) or anti-inflammatory (M2) phenotypes. (Wulandari et al., 2026). PMA was made by dissolving 5 mg of PMA powder in 1.5 ml of water that is free from endotoxins. The specimen was maintained at a temperature of -20 °C, shielded from light, and precautions were taken to prevent repeated freeze-thaw cycles. Injected 1 microliter of the pre-made (PMA) solution into a population of 10cell of U937 Monocyte cell lines and allowed them to grow at a temperature of 37 degrees Celsius for 24 hours (Sharp, 2013).

AMO were resuspended in Dulbecco's Modified Eagle Medium (DMEM) and introduced to the macrophages at a 1:20 ratio. To facilitate optimal parasite internalization by the host cells, all assays were prepared in triplicate and the plates were incubated for 24 hours at 37°C under a 5% CO<sub>2</sub> atmosphere (Hassan & Ali, 2020).

### **Preparation of NTZ and PTX Solutions**

NTZ (CAS No:55981-09-4 *MCE*) was solubilized in DMSO at concentration 50mg/ml (stock solution 5mg/1.08ml) and stored at -20°C for 3 months (Valladares-Méndez et al., 2017). Concentrations of (12.5, 25, 50, 100, 200) µg/ml are used in study.

PTX powder (CAS No: 6493-05-6 *MCE*) (278.3 mg) was weighed and dissolve in 10 mL of sterile PBS or sterile deionized water then vortex until the solution is completely clear (Indrayanto et al., 1998) (Fonseca et al., 2025). Concentrations of (62.5, 125, 250, 500, 1000) µg/ml are used in study.

### **Determination of Cytotoxicity of NTZ and PTX by MTT Assay**

The MTT assay is a highly efficient technique for assessing cell viability and identifying an appropriate and safe dosage of medication. This test employs a colorimetric technique to ascertain the level of toxicity and growth-inhibiting effects of cells. In a darkened room, 5 mg of MTT powder (tetrazolium salt) was dissolved in 1 mL of the PBS solution to make the MTT solution. In addition,  $5 \times 10^5$  PRO per mL were added to 96-well culture plates with various doses of the drugs. After a 72-hour incubation in the dark, 20 microliters of the prepared MTT solution were placed in each well. Then, the cells were centrifuged, and their supernatant was removed. The incubation was repeated for 5 hours at room temperature, and then the wells of the plate received 100 µL of DMSO, and the plate was placed into an ELISA reader with a wavelength of 540 nm to determine the absorbance of each well (Dalimi et al., 2022).

### **Griess Assay for Nitric Oxide Determination**

Nitrite concentrations in the culture supernatants were quantified via the Griess assay. The working Griess reagent was prepared by combining equal volumes of Reagent A (1% [w/v] sulphanilamide) and Reagent B (0.1% [w/v] naphthyl-ethylene-diamine dihydrochloride in 2.5% [w/v] phosphoric acid). For the procedure, 100 µl of each supernatant was combined with 100 µl of the Griess reagent mixture. Following a 10-minute incubation period at room temperature, the absorbance was recorded at 540 nm utilizing a Synergy 4 microplate reader (Biotek, GE). Nitrite levels were subsequently determined by extrapolating from a sodium nitrite (NaNO<sub>2</sub>) standard curve (Mazza et al., 2026).

### **Gene Expression of iNOS and Housekeeping Gene GAPDH by RT-qPCR**

"RT-qPCR" is the term used to refer to the fusion of quantitative (real-time) PCR with reverse transcription PCR. With reverse transcriptase, complementary DNA (cDNA) may be produced using an RNA template by reverse transcription polymerase chain reaction (PCR). RNA is totally extracted using FavorPrep™ Blood/Cultured Cell Total RNA Kit (Table 1) and the primers of gene expression experiment (Table 2):

|   | Material                                     | Cat. No.    | Company        |
|---|----------------------------------------------|-------------|----------------|
| 1 | GoTaq® qPCR System                           | A6001       | Promega/USA    |
| 2 | Primers                                      | ---         | Macrogen/Korea |
| 3 | FavorPrep™ Blood/Cultured Cell Total RNA Kit | FABRK 001-1 | FAVORGEN/Korea |
| 4 | AddScript cDNA Synthesis Kit                 | 22701       | Addbio/Korea   |

Table (1) Materials of RNA Kit

| Species      | Gene  | Primer name | 5'-3'                | Size (bp) | Accession number | Reference             |
|--------------|-------|-------------|----------------------|-----------|------------------|-----------------------|
| Homo sapiens | iNOS  | F           | CAGCGGGATGACTTTCCAA  | 75        | NM_000625.4      | (Zuniga, 2018)        |
|              |       | R           | AGGCAAGATTTGGACCTGCA |           |                  |                       |
| Homo sapiens | GAPDH | F           | GGAGTCAACGGATTGGT    | 206       | NM_002046.7      | (Hamzah et al., 2026) |
|              |       | R           | GTGATGGGATTTCCATTGAT |           |                  |                       |

Table (2) Primers of gene expression experiment

## Statistical analyses

All statistical analyses were conducted using GraphPad Prism (version 10.0, GraphPad Software, Boston, MA, USA). For multi-group comparisons, one-way ANOVA followed by Tukey's [or Dunnett's] post-hoc test were performed. Data were presented as  $\pm$  standard deviation (SD), and differences were considered statistically significant at value  $\leq 0.05$ .

CompuSyn version 1.0 was used to determine combination index (CI) and Dose reduction index (DRI). The combinations at non-constant ratio were analyzed using CompuSyn software (CompuSyn Inc., Paramus, NJ, USA) (Chou, 2008).

## Results

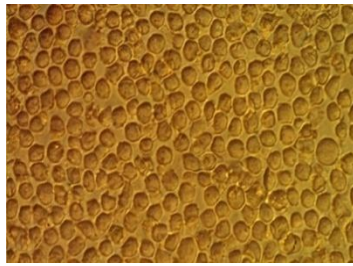
### Differentiation of U937 Monocytes into Macrophage-like Cell Line

The U937 monocytes, which are in suspension form as shown in (Fig. 1).

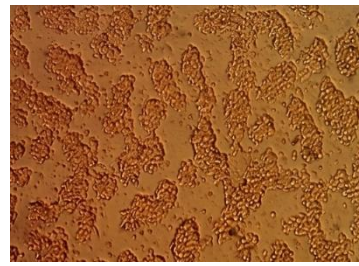


Fig. (1) U937 Monocyte Cell line before treated with PMA (suspension).

Upon treatment with one microliter of PMA when kept at 37 degrees Celsius for a full day developed pseudopods, a macrophage-like morphology, and adhesion (Bakheit et al., 2024) (Uvarova et al., 2024), (see Fig. 2 a & b).



a



b

Fig. (2) U937 Macrophage cell line treated with 1 microliter PMA and kept at 37 degrees Celsius for a full day (adhesion). (a: powered by 40; b powered by 10)

PMA triggers the activation of calcium and phospholipid-dependent type of protein kinase c leading to activation of cAMP metabolism and results in maturation of macrophage like cells (Otte et al., 2011). Microscopic examination can be used to distinguish between physical properties of monocytes and macrophages (Nascimento et al., 2022) as shown in (Fig.1., 2. a & b). U937 macrophage Cell line is infected with promastigote (20:1) incubation 24hr at 34°C, 5% CO<sub>2</sub>, and 95% relative humidity (see Fig. 3).

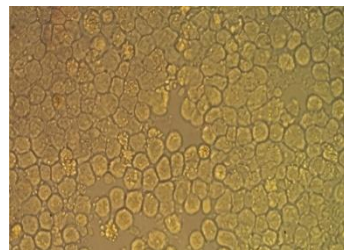
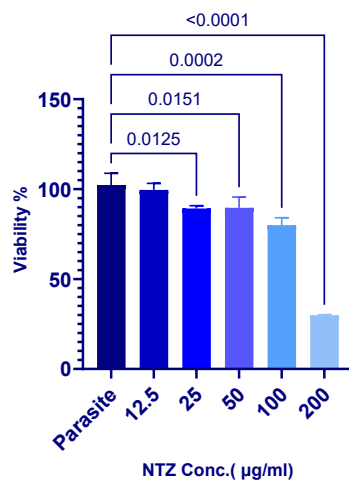


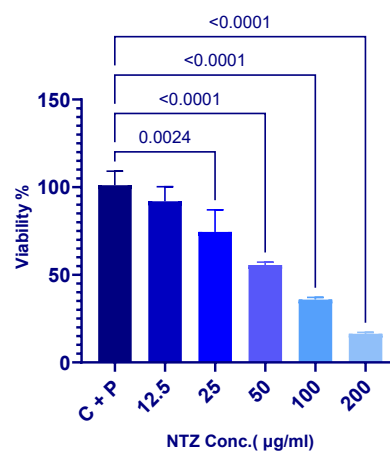
Fig. (3) U937 macrophage Cell line after infected with promastigote (20:1) incubation 24hr at 34°C, 5% CO<sub>2</sub>, and 95% relative humidity.

### The Viability Effects of NTZ and PTX on *L. Tropica*

The cytotoxicity of NTZ on U937 macrophages was evaluated by quantifying it during a 24-hour incubation at 37°C with NTZ doses of (12.5, 25, 50, 100 & 200) µg/ml (see Fig. 4 a & b).



(a)



(b)

Fig. (4) The viability effect of NTX at concentrations of (12,5, 25, 50, 100 & 200) µg/ml, three replicates for each conc. on (a) *L. tropica* promastigote and (b) U937 macrophage infected with *L. tropica* cell line.

The cytotoxicity of PTX on U937 macrophages was evaluated by quantifying it during a 24-hour incubation at 37°C with PTX doses of (62.5, 125, 250, 500 & 1000) µg/ml (see Fig. 5 a & b).

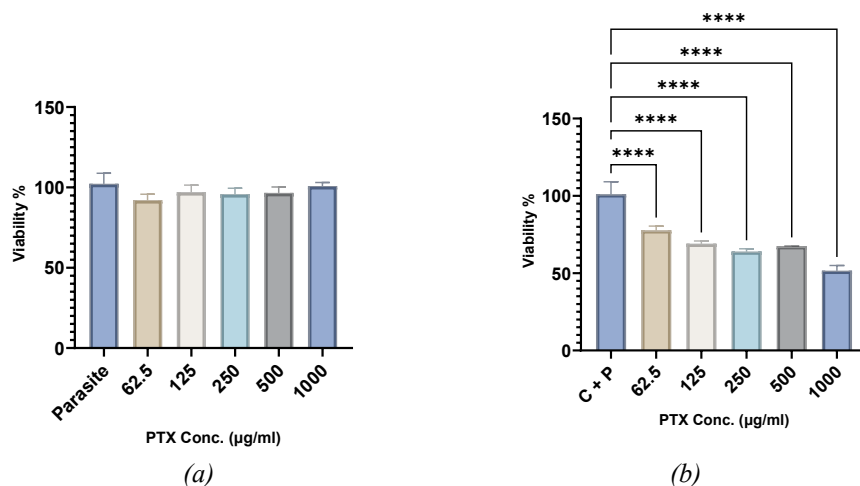


Fig. (5) The viability effect of PTX at concentrations of (62.5, 125, 250, 500 & 1000) µg/ml, three replicates for each conc. on (a) *L. tropica* promastigote; and (b) U937 macrophage infected with *L. tropica* cell line.

The cytotoxicity of combination of PTX 250 µg/ml + NTX (12.5, 25, 50, 100, 200) µg/ml on U937 macrophages infected with *L. tropica* was evaluated by quantifying it during a 24-hour incubation at 37°C (see Fig. 6).

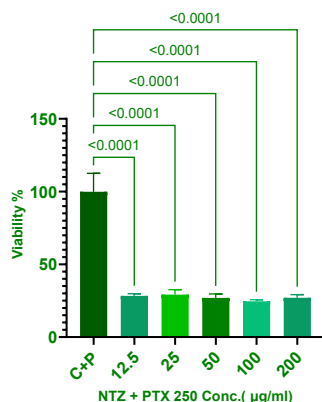


Fig. (6) The viability effect of the combination of PTX 250 µg/ml + NTX (12.5, 25, 50, 100, 200) µg/ml, three replicates for each conc. on U937 macrophage infected with *L. tropica* cell line.

## Synergistic Combinations Activity against Intracellular AMO

The Chou-Talalay combination index CI method at non constant combination was used in this study to investigate the possible synergistic effects of NTZ when combined with PTX against intracellular AMO *L. tropica* in U937 macrophage cell line (see Table 3 & 4) (Fig 7 a & b and Fig 8).

| Drug                  | Dose<br>μg/ml | Effect | X-int   | Y-int                     | m                         | Dm      | R       |
|-----------------------|---------------|--------|---------|---------------------------|---------------------------|---------|---------|
| NTZ                   | 12.5          | 0.1566 | 1.80530 | -2.2535<br>+/-<br>0.26734 | 1.24829<br>+/-<br>0.15263 | 63.8707 | 0.97830 |
|                       | 25            | 0.1905 |         |                           |                           |         |         |
|                       | 50            | 0.3509 |         |                           |                           |         |         |
|                       | 100           | 0.6449 |         |                           |                           |         |         |
|                       | 200           | 0.8349 |         |                           |                           |         |         |
| PTX                   | 62.5          | 0.2335 | 3.01611 | -1.1519<br>+/-<br>0.19578 | 0.38190<br>+/-<br>0.08039 | 1037.80 | 0.93950 |
|                       | 125           | 0.3162 |         |                           |                           |         |         |
|                       | 250           | 0.4255 |         |                           |                           |         |         |
|                       | 500           | 0.3979 |         |                           |                           |         |         |
|                       | 1000          | 0.4891 |         |                           |                           |         |         |
| PTX<br>250μg +<br>NTZ | 12.5          | 0.7203 |         |                           |                           |         |         |
|                       | 25            | 0.7089 |         |                           |                           |         |         |
|                       | 50            | 0.7312 |         |                           |                           |         |         |
|                       | 100           | 0.7545 |         |                           |                           |         |         |
|                       | 200           | 0.7301 |         |                           |                           |         |         |

Table 3: Dose response relationship and median effect parameters of NTZ, PTX, and their combination.

m: is the slope of the median effect plot which indicate the shape of dose response curve.

Dm: is the median effect dose.

r: is a linear correlation coefficient.

Dm, m, CI parameters for the (NTZ+PTX) combinations were obtained using the ComboSyn software.

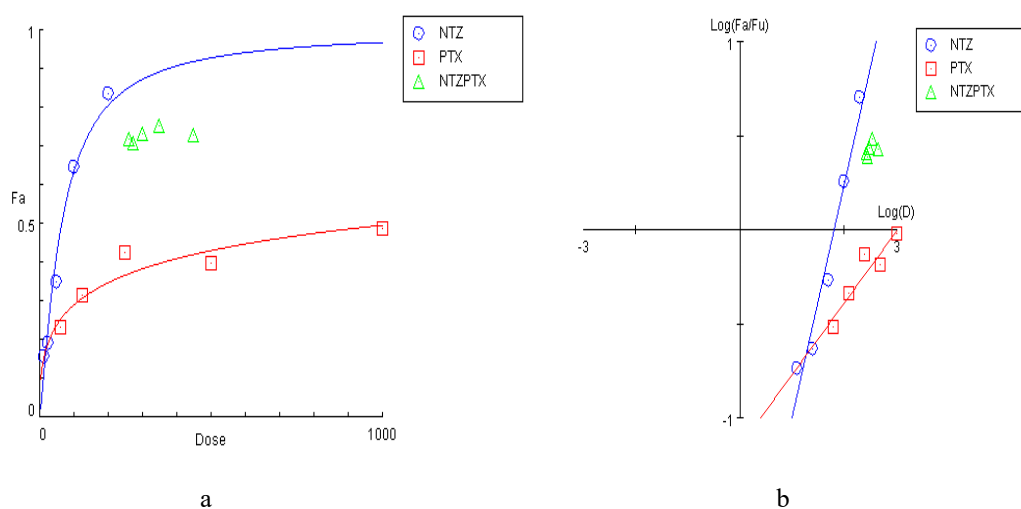


Fig (7) a. Dose-Effect Curve for NTZ and PTX alone and in combination.

b. Median-Effect Plot for NTZ and PTX alone and in combination

| NTZ dose $\mu\text{g/ml}$ | PTX dose $\mu\text{g/ml}$ | Effect | CI                         |
|---------------------------|---------------------------|--------|----------------------------|
| 12.5                      | 250.0                     | 0.7203 | 0.11196 strong synergism   |
| 25.0                      | 250.0                     | 0.7089 | 0.21528 strong synergism   |
| 50.0                      | 250.0                     | 0.7312 | 0.36869 moderate synergism |
| 100.0                     | 250.0                     | 0.7545 | 0.64965 moderate synergism |
| 200.0                     | 250.0                     | 0.7301 | 1.42874 antagonism         |

Table 4: CI Data for Non-Constant Combo (NTZ+PTX) in Chou-Talalay method, CI dictate the nature of drug interaction. Dose dependent effects were analyzed by CompuSyn software: CI <1 Synergism, CI =1 Additive, CI >1 Antagonism. very strong synergism (CI < 0.1), strong synergism (CI = 0.1–0.3), synergism (CI = 0.3–0.7), moderate synergism (CI = 0.7–0.85), slight synergism (CI = 0.85–0.90), nearly additive (CI = 0.90–1.10), slight antagonism (CI = 1.10–1.20), moderate antagonism (CI = 1.20–1.45), antagonism (CI = 1.45–3.30), strong antagonism (CI = 3.30–10), very strong antagonism (CI > 10) (Chanmol et al., 2022).

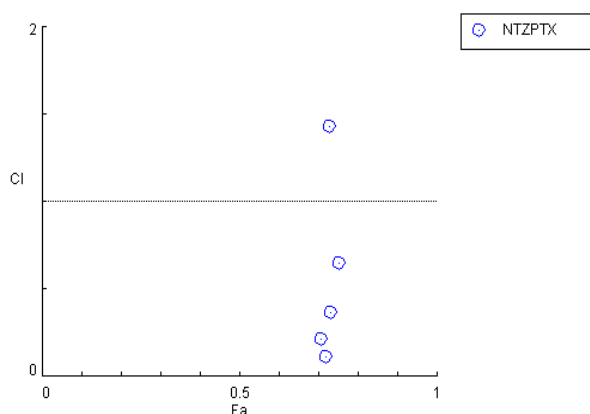


Fig. (8) Combination Index Plot.

The Chou-Talalay Dose reduction index (DRI) method at non constant combination NTZ+PTX was used in this study to represent how many folds the dose of each drug can be reduced when used in combination compared to monotherapy (see Table 5 & Fig. 9).

| Fa     | Dose NTZ $\mu\text{g/ml}$ | Dose PTX $\mu\text{g/ml}$ | DRI NTZ | DRI PTX |
|--------|---------------------------|---------------------------|---------|---------|
| 0.7203 | 136.273                   | 12354.7                   | 10.9018 | 49.4186 |
| 0.7089 | 130.305                   | 10672.3                   | 5.21219 | 42.6893 |
| 0.7312 | 142.385                   | 14259.8                   | 2.84770 | 57.0391 |
| 0.7545 | 157.008                   | 19628.8                   | 1.57008 | 78.5151 |
| 0.7301 | 141.749                   | 14052.6                   | 0.70874 | 56.2104 |

Table 5: DRI data for non-constant Combo (NTZ+PTX): DRI>1 synergism, DRI =1 additive and DRI <1 antagonism.

Fa: the fraction affected.

Doses: represent theoretical monotherapy to achieve that specific fa.

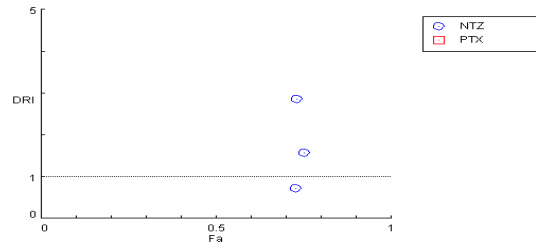


Fig. (9) DRI Plot for Non-Constant Combo: (NTZ+PTX).

The normalized isobologram (Chou-Chou plot) is a unit less, two-dimensional graphical model used in pharmacology to evaluate the interaction between two or more drugs (for the non-constant ratio combination design of NTZ+PTX) (see Fig. 10).

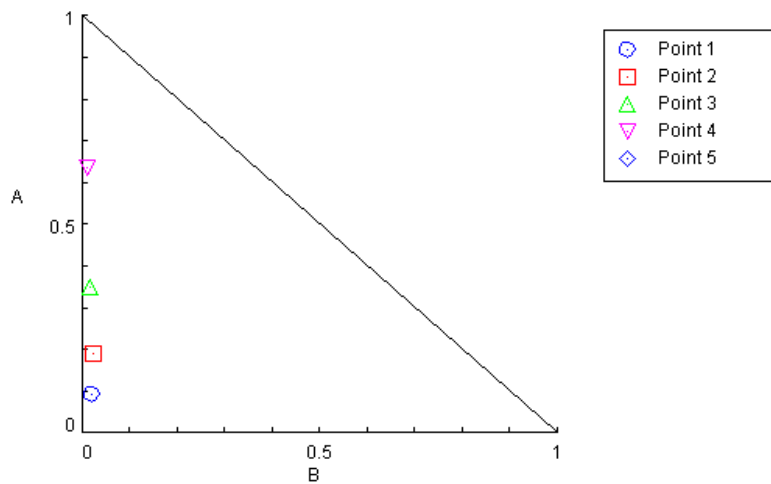


Fig. (10) The normalized isobologram (Chou-Chou plot) for the non-constant ratio combination design of (NTZ+PTX):

- Axis B (x-axis) is PTX, because combination allowed for a massive >40-fold dose reduction for PTX, its normalized dose approaches zero.
- Axis A (y-axis) is NTZ The vertical spread of the points entirely reflects escalating doses of NTZ.

### Measuring iNOS Gene Expression in U937 by Real-time qPCR

The iNOS gene expression was examined by qPCR to decide on the expression in uninfected, infected non-treated, and infected treated cells in different concentrations of NTX, PTX alone and at selected non constant ratio combinations and the nitrites found in the supernatants was assessed using the Griess assay (as shown in Fig 11, 12, and 13).

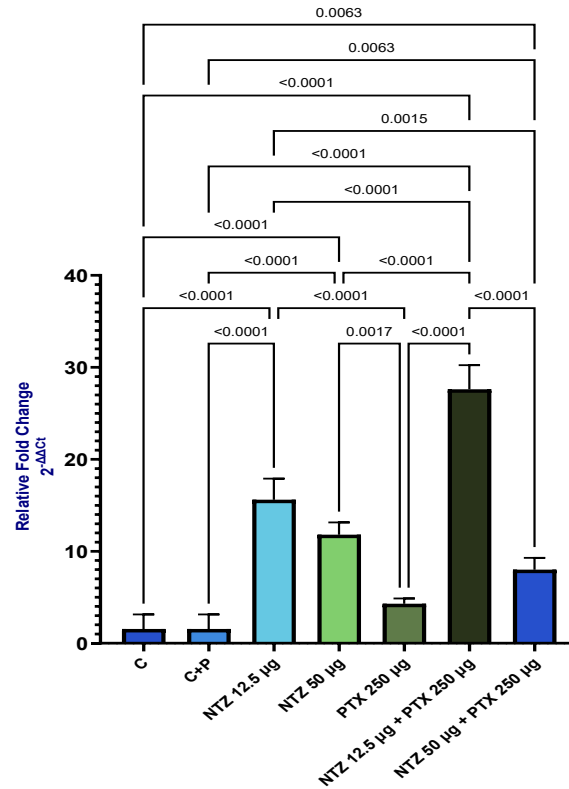


Fig. (11) Impact of NTX (12.5, 50) µg/ml, PTX (250 µg/ml), combination of (PTX 250 + NTZ 12.5) µg/ml and combination of (PTX 250 + NTZ 50) µg/ml on expression of iNOS gene in U937 after 24 h incubation.

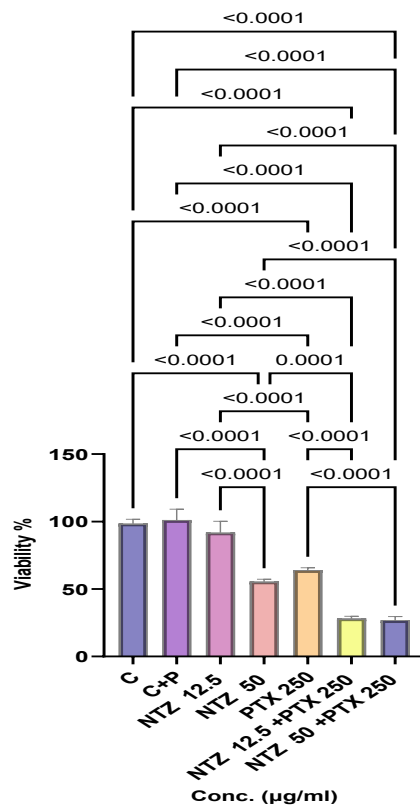


Fig. (12) Impact of NTX (12.5, 50)  $\mu\text{g/ml}$ , PTX (250  $\mu\text{g/ml}$ ), combination of (PTX 250 + NTZ 12.5)  $\mu\text{g/ml}$  and combination of (PTX 250 + NTZ 50)  $\mu\text{g/ml}$  on viability in U937 macrophage infected with *L. tropica* after 24 h incubation.

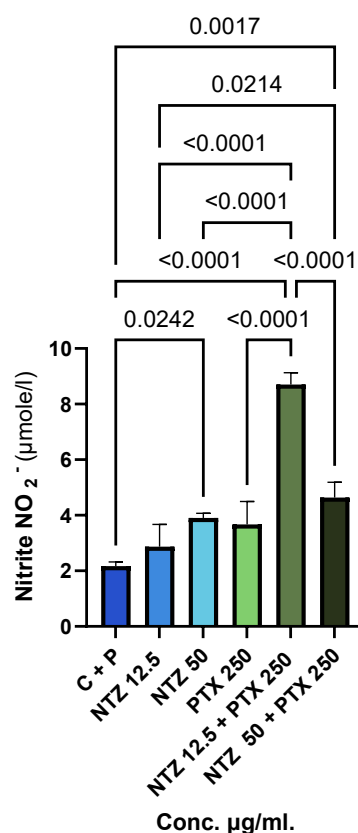


Fig. (13) Impact of NTX (12.5, 50)  $\mu\text{g/ml}$ , PTX (250  $\mu\text{g/ml}$ ), combination of (PTX 250 + NTZ 12.5)  $\mu\text{g/ml}$  and combination of (PTX 250 + NTZ 50)  $\mu\text{g/ml}$  on nitrite  $\text{NO}_2^-$  level.

## Discussion

This study evaluated the cytotoxicity of NTZ on PRO *L. tropica* and U937 macrophage infected with *L. tropica* at concentrations of (12.5, 25, 50, 100 & 200)  $\mu\text{g/ml}$ , three replicates for each concentration (3N) as shown in Fig.4 a & b. The results suggest a highly significant reduction in *L. tropica* cell line growth ( $p < 0.05$ ) as compared with the control group (c+p).

Previous research has provided three main mechanisms for the antileishmanial activity of NTZ. The first seems to involve the disruption of the electron transfer reaction mediated by pyruvate: ferredoxin oxidoreductase (PFOR), a pathway crucial for anaerobic energy metabolism. Additionally, cellular energy generation relies on pyruvate kinase, a key enzyme in the glycolytic cascade that catalyzes the synthesis of ATP (Gurgen et al., 2011) and (Bashir et al., 2023). The second mechanism is done by the inhibition of the disulfide isomerase protein (ALBALAWI et al., 2021). In the third mechanism, NTZ demonstrates the potential to circumvent drug resistance arising from enzymatic mutations by blocking the primary step of the metabolic pathway. Specifically, it impedes the binding of pyruvate to the thiamine pyrophosphate cofactor, rather than competing directly with downstream cellular substrates (Khalid et al., 2022). NTZ has leishmanicidal activity on PRO and AMO forms of *L. species* through the successful repurposing of existing drugs for leishmaniasis, which agrees with (Lye et al., 2024).

While the cytotoxicity of PTX on PRO *L. tropica* at concentrations of (62.5, 125, 250, 500 & 1000)  $\mu\text{g/ml}$  suggests no significant reduction on PRO, it suggests significant reduction in U937 macrophage infected with *L. tropica* growth ( $p < 0.05$ ) compared with the control group as shown in Fig. 5 a & b. In vitro, there is no evidence that PTX directly kills *Leishmania* species but its major contribution is the reduction of the TNF- $\alpha$  mediated tissue damage (Weatherhead & Woc-Colburn, 2015). This is because PTX exercises anti-inflammatory effects by inhibiting several cytokines, among which are TNF- $\alpha$ , IL-1, and IL-6 which participate in a lot of inflammatory skin diseases (Balazic et al., 2023).

The cytotoxicity of the combination of PTX 250  $\mu\text{g/ml}$  + NTX (12.5, 25, 50, 100, 200)  $\mu\text{g/ml}$  on U937 macrophage infected with *L. tropica* suggests highly significant reduction in *L. tropica* cell line growth at all concentrations compared with the control group (see Fig 6), which agrees with Balazic et al's study which states that a therapy of PTX combined with pentavalent antimony (PA) agents is expected to boost parasites destruction in cutaneous leishmaniasis, degrading tissue damage and inflammation (Balazic et al., 2023).

The Chou-Talalay' method of combination index CI at non-constant combination was used in this study to investigate the potential synergistic effects of NTZ when added to PTX against intracellular AMO *L. tropica* in U937 macrophage cell line. PTX was maintained at constant dose of 250  $\mu\text{g/ml}$  while the dose of NTZ was progressively increased from (12.5 to 200)  $\mu\text{g/ml}$ . To characterize the pharmacological interactions as synergistic, additive, or antagonistic, the combination index (CI) for each treatment pairing was calculated based on the percentage of growth inhibition relative to the untreated control. as shown in Table 3. The biological efficacy, represented by the fraction affected (fa), remained relatively stable across all combination ratios, demonstrating a narrow effect plateau between 0.7089 and 0.7545. Despite this stagnant efficacy, CI analysis revealed a highly dose dependent profile.

m is the slope of the median effect plot or kinetic order which indicates the shape of dose response curve: the value for NTZ equal to 1.24 ( $m > 1$ ) and the shape is sigmoidal, while the value for PTX equal to 0.38 ( $m < 1$ ) and the shape is flat sigmoidal. Dm is the potency of drug or median effect dose. A lower Dm value indicates more potent drug, the value for NTZ equal to 63.87, making it substantially more potent than PTX with Dm value equal to 1037.8.

r is a linear correlation coefficient which measures the goodness of fit. The closer the r value to 1, the more reliable it becomes. The r value of NTZ is 0.97 while it is 0.93 for PTX, which indicates that the data point fits the linearized model very well as shown in Table 3 and Fig 7 a & b.

In Chou-Talalay method, CI dictates the nature of drug interaction. Dose dependent effects are analyzed by CompuSyn software. At lower NTZ concentrations (12.5 and 25)  $\mu\text{g/ml}$ , the combination yielded CI values of 0.11196 and 0.21528 respectively, indicating strong synergism. As the NTZ dose was increased to (50.0 and 100.0)  $\mu\text{g/ml}$ , the interaction weakened to moderate synergism (CI values of 0.36869 and 0.64965, respectively). Ultimately, at the highest NTZ concentration evaluated 200  $\mu\text{g/ml}$ , the interaction transitioned into antagonism, producing a CI value of 1.42874. This indicates that while the low-dose NTZ/high-dose PTX ratio is highly synergistic and efficient at clearing the parasite, excessive NTZ concentrations negatively impact the combination's overall efficiency without providing additional therapeutic benefit as shown in Table 4 and Fig. 8. (Pinto et al., 2020) state that, through its active metabolite tizoxanide, NTZ at lower doses (12.5 and 25)  $\mu\text{g/ml}$  primarily acts by inhibiting the PFOR enzyme pathway essential for parasite energy metabolism. These specific enzymatic targets within the *Leishmania* parasites are adequately saturated, operating synergistically with the constant dose of PTX. Increasing the NTZ beyond this dose provides no additional antiparasitic efficacy.

DRI is a pharmacological metric used in Chou-Talalay method for analysis of drugs combinations by measuring how much the dose of a single drug can be reduced when used in combination with another drug as shown in Table 5 and Fig. 9. Optimal synergy (low dose) is at the fa of 0.7203 (which corresponds to 12.5  $\mu\text{g/ml}$  NTZ combination dose) and the DRI NTZ is 10.9018. This means that pairing NTZ with

PTX achieves a 72% parasite clearance, which equals nearly 11 times less dose of NTZ than it would normally need to alone. This is highly favorable. As NTZ combination dose increases (25, 50, 100)  $\mu\text{g/ml}$ , the DRI steadily drops to 5.2, 2.8, and 1.5. The dose reduction benefit is eroding. At the highest dose (fa of 0.7301, corresponding to 200 $\mu\text{g/ml}$  NTZ combination dose), the DRI NTZ drops to 0.70874 (which is  $< 1$ ). This means that antagonism and cellular pathway interference have occurred.

The normalized isobologram (Chou-Chou plot) for the non-constant ratio combination design of (NTZ+PTX) ties the mathematical findings and biological mechanisms together. The diagonal line connecting 1 on the y-axis to 1 on the x-axis, is the line of additivity ( $\text{CI} = 1$ ). Any point plotting below and to the left of this line is synergistic. Any point plotting above and to the right is antagonistic. All data points (1, 2, 3, 4) are hugging the far-left side of the graph. This is not a glitch; it is a brilliant visual confirmation of DRI (see Fig. 10). Point 5 (representing 200 NTZ dose) is missing from this bounded area because its normalized dose is roughly 1.41 (derived from its DRI of  $< 1$ ). It falls entirely off the top of this standard scale (lies above the line of additivity).

This normalized isobologram shows that combining a low dose of NTZ with PTX allows for a highly synergistic, macrophage-friendly treatment, but continuing to push the NTZ dose higher drives the interaction upward toward additivity and eventual antagonism.

iNOS gene expression was explored via qPCR to identify the expression in uninfected, infected non-treated and infected treated cells in different concentrations of NTX, PTX alone and at selected non constant ratio combinations; then, the presence of nitrites in the supernatants was evaluated using the Griess assay (see Fig 11 and Fig 13).

There is no significant difference in iNOS expression between the uninfected control (C) and the infected control (C+P) associated with low level of nitrite. This indicates that the parasite alone inhibits a strong, spontaneous iNOS response in the macrophages. Previous research has provided three main reasons for such an inhibition. First, because the expression of iNOS is directly correlated with leishmanicidal activity of the macrophages, the iNOS regulatory activity of lipophosphoglycan (LPG) is contained within the PG moiety, a predominant surface molecule of PRO (Neamah et al., 2023). Secondly, *Leishmania* is capable of suppressing iNOS expression via various mechanisms, notably by disrupting the JAK2-STAT1 and ERK1/2-AP1 signaling cascades (Palomino-Cano et al., 2024). Third, Thirdly, iNOS expression is subject to post-transcriptional regulation by microRNAs (miRNAs)—short, non-coding RNAs that bind to the 3' untranslated regions (3' UTRs) of target messenger RNAs, inducing their degradation and inhibiting translation. Specifically, *Leishmania* infection has been shown to dysregulate miR-30, miR-294, and miR-721; these miRNAs directly target the iNOS transcript, thereby depleting intracellular iNOS levels (Calegari-Silva et al., 2015).

Both NTZ (12.5 and 25.0)  $\mu\text{g/ml}$  and PTX (250  $\mu\text{g/ml}$ ) independently upregulate iNOS expression when compared to the infected control (C+P), with all showing high statistical significance ( $p < 0.0001$ ). NTZ alone at both (12.5 and 50)  $\mu\text{g/ml}$  is a notably stronger inducer of iNOS than PTX alone (250  $\mu\text{g/ml}$ ). This upregulation of iNOS expression is due to the direct parasite targeting of NTZ through inhibition of PFOR and energy metabolism pathways (Bashir et al., 2023) which leads to improving the spontaneous iNOS gene expression. However, the upregulation happened with slight increase in the nitrite level the weapon of gene at NTZ 12.5  $\mu\text{g/ml}$  and with significant increase ( $p$  value  $< 0.05$ ) at NTZ 50  $\mu\text{g/ml}$  (see Fig11 and Fig.13). Previous research state that L-arginine affects the parasite survival through the production of polyamines by both *Leishmania* (La-arginase) and macrophage arginases (Da Silva et al., 2012). L-arginine is essential for nitric oxide (NO) synthesis via NOS2 (iNOS), a pathway critical for driving the microbicidal inflammatory response in macrophages. However, even when host iNOS is adequately expressed, *Leishmania* can subvert this defense mechanism by upregulating its own arginase, which competitively depletes the shared L-arginine pool and blunts NO generation (Wilkins-Rodríguez et al., 2020). Conversely, the absence of parasitic arginase alters host transcriptional profiles, leading to a distinct upregulation of iNOS mRNA. Consequently, inhibiting parasite arginase successfully redirects

L-arginine metabolism toward citrulline and NO synthesis, significantly enhancing the host's capacity to control the infection (Muxel et al., 2017).

NTZ, in contrast, downregulated the expression of iNOS, while simultaneously limiting the release of inflammatory cytokines, including TNF- $\alpha$ , IL-1 $\beta$ , and IL-6 (Abo Elqasem et al., 2026) which is seen in decreasing iNOS gene expression between (12.5 and 50)  $\mu\text{g/ml}$  of NTZ but associated with decreasing parasite growth (see Fig11 and Fig12). PTX has been proposed as a strategy for improving the therapeutic response in leishmaniasis by the modulation of IFN- $\gamma$  and TNF- $\alpha$  production (Castro et al., 2022).

The first combination (NTZ 12.5 + PTX 250)  $\mu\text{g/ml}$  yields the highest peak on the graph, resulting in an approximate 27 to 28-fold increase in iNOS expression. Crucially, this response is significantly higher than the effects of either drug acting alone at those same concentrations with significant differences when compared to NTZ 12.5 alone and PTX 250 alone ( $p < 0.0001$ ). Because the combined effect is substantially greater than the sum of the individual effects, this strongly points to a synergistic interaction at this specific non-constant ratio. PTX potentiates the antileishmanial effects of low dose of NTZ. (Cincura et al., 2022) state that the association of PTX, as TNF inhibitor, with antimony lessens the healing period and the rates of therapeutic failure. Thus, it can be the first-choice therapy. In addition to accompanying the severity of the disease, TNF also takes part in the pathogenesis of leishmania.

Conversely, pairing the higher dose of NTZ (50  $\mu\text{g/ml}$ ) with PTX (250  $\mu\text{g/ml}$ ) results in a dramatic decrease in iNOS expression compared to using NTZ 50  $\mu\text{g/ml}$  alone. It is also significantly lower than the lower-dose NTZ combination ( $p < 0.0001$ ). This suggests a specific high-dose combination may be triggering cytotoxicity in the U937 cells, thereby shutting down overall cellular metabolism and transcriptional machinery, leading to the drop in measured iNOS mRNA. This is associated with significant reduction in viability and significant reduction in nitrite level as compared with the first combination (see Fig. 11, Fig. 12 and Fig. 13).

Finally, the non-constant ratio combination of NTZ 12.5  $\mu\text{g/ml}$  + PTX 250  $\mu\text{g/ml}$  is found to be the optimal therapeutic window in this model since it successfully triggers a massive upregulation of iNOS gene expression, which is essential for producing the nitric oxide in the form nitrite required for macrophage-mediated clearance of the parasite. In addition to the direct leishmaniacidal effect of NTZ on parasite, it is found that this combination enhances the immunomodulation effects of the two drugs, a finding supporting Sampaio's study which asserts that the antileishmanial drugs with an immunomodulatory agent can boost immunomodulation, kill parasites, and directly modulate host immunity (Sampaio, 2023).

## Conclusion

This study demonstrates the significant antileishmanial activity of non-constant ratio combination of NTZ and PTX and highlights the correlation between iNOS gene expression and nitrite ( $\text{NO}_2^-$ ) released by macrophages upon exposure to *L. tropica*. Finally, adopting combination therapy models serves as a robust strategy to overcome the resistant limitations of anti-leishmanial monotherapies.

## References

- Abadías-Granado, I., Diago, A., Cerro, P., Palma-Ruiz, A., & Gilaberte, Y. (2021). Cutaneous and mucocutaneous leishmaniasis. *Actas Dermo-Sifiliográficas (English Edition)*, 112(7), 601–618.
- Abbas, T. F., & Ali, H. Z. (2024). Lupeol stimulates iNOS, TNF- $\alpha$ , and IL-10 expression in the U937 cell line infected with old-world *Leishmania donovani*. *Cytokine*, 183, 156757.
- Abo Elqasem, A. A., El-Menyawy, H. M., Ahmed Amer, D. A.-F., & El-Wakil, E. S. (2026). Antiparasitic, anti-inflammatory, and immunomodulatory effects of *Portulaca oleracea* against *Cryptosporidium parvum* in experimentally infected immunosuppressed mice. *Beni-Suef University Journal of Basic and Applied Sciences*, 15(1), 67.
- Aksoy, T., & Kilimcioğlu, A. A. (2025). Thymol's antileishmanial activity and its impact on host cytokine profiles: In vitro and ex vivo studies on *Leishmania tropica*. *Parasitology International*, 103139.
- Al-Kuraishy, H. M., Al-Gareeb, A. I., Elekhawwy, E., & Batiha, G. E.-S. (2022). Nitazoxanide and COVID-19: a review. *Molecular biology reports*, 49(11), 11169–11176.
- Al Zadawi, K. A. M. (2022). Geopolitical and Geospatial Conflicts Affecting Cutaneous. *Leishmaniasis: General Aspects of a Stigmatized Disease*, 113.
- ALBALAWI, A. E., ALMOHAMMED, H. I., KHALAF, A. K., D ALANAZI, A., MOGHADDAM, A., NIAZI, M., & MAHMOUDVAND, H. (2021). The Potential Use of Nitazoxanide in the Treatment of Cutaneous Leishmaniasis: In-Vitro Assays Against *Leishmania Tropica*. *International Journal of Pharmaceutical Research*, 13(2).
- Bakheit, H. F., Taurin, S., Elamin, E. M., & Bakhiet, M. (2024). Akt1 players promote PMA U937 cell line differentiation into macrophage-like cells. *Arab Gulf Journal of Scientific Research*, 42(4), 1257–1270.
- Balazic, E., Axler, E., Konisky, H., Khanna, U., & Kobets, K. (2023). Pentoxifylline in dermatology. *Journal of Cosmetic Dermatology*, 22(2), 410–417.
- Bashir, S., Shabbir, K., ud Din, F., Khan, S. U., Ali, Z., Khan, B. A., Kim, D. W., & Khan, G. M. (2023). Nitazoxanide and quercetin co-loaded nanotransfersomal gel for topical treatment of cutaneous leishmaniasis with macrophage targeting and enhanced anti-leishmanial effect. *Heliyon*, 9(11).
- Bhattacharyya, A., Chhajera, R., Kamran, M., Ejazi, S. A., Didwania, N., Gayen, S., da Silva, F. O., Costa, D. L., de Mendonca, I. L., & Costa, C. H. N. (2026). Enhanced diagnostic tools for visceral leishmaniasis using kinase based antigens in Brazilian human and canine samples. *Scientific Reports*.
- Brito, G., Dourado, M., Polari, L., Celestino, D., Carvalho, L. P., Queiroz, A., Carvalho, E. M., Machado, P. R., & Passos, S. (2014). Clinical and immunological outcome in cutaneous leishmaniasis patients treated with pentoxifylline. *The American journal of tropical medicine and hygiene*, 90(4), 617.
- Calegari-Silva, T. C., Vivarini, Á. C., Miqueline, M., Dos Santos, G. R., Teixeira, K. L., Saliba, A. M., de Carvalho, S. N., de Carvalho, L., & Lopes, U. G. (2015). The human parasite *Leishmania amazonensis* downregulates iNOS expression via NF- $\kappa$ B p50/p50 homodimer: role of the PI3K/Akt pathway. *Open biology*, 5(9), 150118.
- Castro, M. d. M., Cossio, A., Navas, A., Fernandez, O., Valderrama, L., Cuervo-Pardo, L., Marquez-Oñate, R., Gómez, M. A., & Saravia, N. G. (2022). Pentoxifylline in the treatment of cutaneous leishmaniasis: A randomized clinical trial in Colombia. *Pathogens*, 11(3), 378.
- Chanmol, W., Siriwasatien, P., & Intakhan, N. (2022). In vitro anti-*Leishmania* activity of 8-hydroxyquinoline and its synergistic effect with amphotericin B deoxycholate against *Leishmania martiniquensis*. *PeerJ*, 10, e12813.

- Chou, T.-C. (2008). Preclinical versus clinical drug combination studies. *Leukemia & lymphoma*, 49(11), 2059–2080.
- Choudhari, V. I., Satpute, S. K., & Pai, K. (2026). Orphan Medications: An Innovative and Promising Approach against Leishmaniasis. *Current Microbiology*, 83(4), 210.
- Cincura, C., Costa, R. S., De Lima, C. M. F., Oliveira-Filho, J., Rocha, P. N., Carvalho, E. M., & Lessa, M. M. (2022). Assessment of immune and clinical response in patients with mucosal leishmaniasis treated with pentavalent antimony and pentoxifylline. *Tropical Medicine and Infectious Disease*, 7(11), 383.
- Cosma, C., Maia, C., Khan, N., Infantino, M., & Del Riccio, M. (2024). Leishmaniasis in humans and animals: A one health approach for surveillance, prevention and control in a changing world. *Tropical Medicine and Infectious Disease*, 9(11), 258.
- Da Silva, M. F. L., Zampieri, R. A., Muxel, S. M., Beverley, S. M., & Floeter-Winter, L. M. (2012). Leishmania amazonensis arginase compartmentalization in the glycosome is important for parasite infectivity. *PloS one*, 7(3), e34022.
- Dalimi, A., Moghadamzad, Z., Jafari, M. M., KarimiPour, S. A., & Pirestani, M. (2022). Assessment of spring leaf extract of artemisia aucheri effects on leishmania tropica/infantum.
- Fonseca, J., Fonseca, S., & Ayala, A. (2025). Top to Down Solubility Modulation of New Pentoxifylline Cocrystals. *Crystal Growth & Design*, 25(23), 10040–10052.
- Gurgen, J., Hogan, D., Grace, E., & Johnson, D. (2011). Nitazoxanide in the treatment of chronic cutaneous leishmaniasis resistant to traditional sodium stibogluconate.
- Hamzah, E. F., Altaee, A. H., & Aljubawii, A. (2026). Integrated protein and gene expression analysis of S100A1 and S100A8/A9 demonstrates coordinated dysregulation of calcium homeostasis, innate immune activation, and angiogenic signaling in acute myocardial infarction.
- Hassan, G. M., & Ali, H. Z. (2020). Ex vivo study of Anti-leishmanial activity of Artemisinin against Leishmania tropica amastigote. *Research Journal of Pharmacy and Technology*, 13(8), 3787–3787.
- Indrayanto, G., Syahrani, A., Moegihardjo, Soeharyono, Lianawati, T., Wahyuningsih, I., Aditama, L., & Brittain, H. G. (1998). Pentoxifylline. In H. G. Brittain (Ed.), *Analytical Profiles of Drug Substances and Excipients* (Vol. 25, pp. 295–339). Academic Press. [https://doi.org/https://doi.org/10.1016/S0099-5428\(08\)60758-3](https://doi.org/https://doi.org/10.1016/S0099-5428(08)60758-3)
- Irshad, H., & Khalid, M. Z. (2022). Types and treatments of leishmaniasis. *Biomedical Journal of Scientific & Technical Research*, 42(5), 34037–34042.
- Keyhani, A., Jafarzadeh, A., Sharifi, I., & Salarkia, E. (2025). Leptin enhances the efficacy of glucantime to modulate macrophage polarization toward the M1 phenotype in Leishmania tropica-infected macrophages. *Parasites & Vectors*, 18(1), 360.
- Khalid, H., Batool, S., ud Din, F., Khan, S., & Khan, G. M. (2022). Macrophage targeting of nitazoxanide-loaded transethosomal gel in cutaneous leishmaniasis. *Royal society open science*, 9(10), 220428.
- Loría-Cervera, E. N., & Andrade-Narvaez, F. (2020). The role of monocytes/macrophages in Leishmania infection: A glance at the human response. *Acta tropica*, 207, 105456.
- Lye, L.-F., Lin, C.-F., Ou, Y.-C., & Chen, C.-M. (2024). Recent advances in antileishmanial drugs: New leishmaniasis medicine through repurposing approach. *Tungs' Medical Journal*, 18(Suppl 1), S1–S5.
- Majoor, A., Michel, G., Marty, P., Boyer, L., & Pomares, C. (2025). Leishmaniasis: Strategies in treatment development. *Parasite*, 32, 18.
- Maksouri, H., Dang, P. M.-C., Rodrigues, V., Estaquier, J., Riyad, M., & Akarid, K. (2017). Moroccan strains of Leishmania major and Leishmania tropica differentially impact on nitric oxide production by macrophages. *Parasites & Vectors*, 10(1), 506.

- Mazza, G., Perego, F., Coletta, S., Proverbio, D., D'Elíos, M. M., Taramelli, D., De Bernard, M., Bruschi, F., & Basilico, N. (2026). Helicobacter pylori Neutrophil Activating Protein (HP-NAP) Enhances the Anti-Leishmanial Activity of Canine Macrophages Against Leishmania infantum. *Pathogens*, 15(2), 184.
- mondiale de la Santé, O., & Organization, W. H. (2023). Global leishmaniasis surveillance, 2022: assessing trends over the past 10 years—Surveillance mondiale de la leishmaniose, 2022: évaluation des tendances des 10 dernières années. *Weekly Epidemiological Record= Relevé épidémiologique hebdomadaire*, 98(40), 471–487.
- Muxel, S. M., Laranjeira-Silva, M. F., Zampieri, R. A., & Floeter-Winter, L. M. (2017). Leishmania (Leishmania) amazonensis induces macrophage miR-294 and miR-721 expression and modulates infection by targeting NOS2 and L-arginine metabolism. *Scientific Reports*, 7(1), 44141.
- Nascimento, C. R., Fernandes, N. A. R., Maldonado, L. A. G., & Junior, C. R. (2022). Comparison of monocytic cell lines U937 and THP-1 as macrophage models for in vitro studies. *Biochemistry and biophysics reports*, 32, 101383.
- Neamah, S. D., Ali, H. Z., & Al-Halbosi, M. M. (2022). Detection of artemisinin effect on macrophage inducible nitric oxide gene expression in macrophage infected with Leishmania donovani. *Annals of Parasitology*, 68(2).
- Neamah, S. D., Ali, H. Z., & Al-Halbosi, M. M. (2023). Artemisinin efficacy on iNOS production in U937 cell-line infected with Leishmania donovani. *Iraqi Journal of Science*, 525–535.
- Nunes, A. C. F., de Araújo Ribeiro, A. L., Aversi-Ferreira, T. A., & Aversi, M. T. M. (2026). WORLD EPIDEMIOLOGY OF LEISHMANIASIS: A NARRATIVE REVIEW. *Revista Tópicos*, 4(32), 1–20.
- Otte, A., Mandel, K., Reinstrom, G., & Hass, R. (2011). Abolished adherence alters signaling pathways in phorbol ester-induced human U937 cells. *Cell Communication and Signaling : CCS*, 9, 20 – 20.
- Palomino-Cano, C., Moreno, E., Irache, J. M., & Espuelas, S. (2024). Targeting and activation of macrophages in leishmaniasis. A focus on iron oxide nanoparticles. *Frontiers in immunology*, 15, 1437430.
- Pinto, E. G., Barbosa, L. R., Mortara, R. A., & Tempone, A. G. (2020). Targeting intracellular Leishmania (L.) infantum with nitazoxanide entrapped into phosphatidylserine-nanoliposomes: An experimental study. *Chemico-Biological Interactions*, 332, 109296.
- Ruiz-Postigo, J. A., Jain, S., Madjou, S., Agua, J. F. V., Maia-Elkhoury, A. N., Valadas, S., Warusavithana, S., Osman, M., Yajima, A., & Lin, Z. (2023). Global leishmaniasis surveillance, 2022: assessing trends over the past 10 years.
- Sampaio, R. N. R. (2023). Pharmacotherapy in leishmaniasis: Old, new treatments, their impacts and expert opinion. *Expert Opinion on Pharmacotherapy*, 24(2), 153–158.
- Sharp, B. M. (2013). Conversion of the U937 Monocyte into " Macrophage-Like" Populations Exhibiting M1 or M2 Characteristics.
- Torres-Guerrero, E., Quintanilla-Cedillo, M. R., Ruiz-Esmenjaud, J., & Arenas, R. (2017). Leishmaniasis: a review. *F1000Research*, 6, 750.
- Uvarova, A., Tkachenko, E., Stasevich, E., Bogomolova, E., Zheremyan, E., Kuprash, D., & Korneev, K. (2024). The rs2564978 (T) allele associated with severe influenza a disrupts the binding site for myeloid differentiation factor PU. 1 and reduces CD55/DAF gene promoter activity in macrophages. *Molecular Biology*, 58(2), 255–265.
- Valladares-Méndez, A., García-Flores, M., Navarrete-Vázquez, G., Orozco-Castellanos, L. M., Hernandez-Núñez, E., & Rivera-Leyva, J. C. (2017). Physicochemical characterization

- of two new Nitazoxanide analogs with antiparasitic activity. *Medicinal Chemistry Research*, 26(1), 9–18.
- Weatherhead, J. E., & Woc-Colburn, L. (2015). Therapeutic options and vaccine development in the treatment of leishmaniasis. *World Journal of Pharmacology*, 4(2), 210–218.
- Wilkins-Rodríguez, A. A., Pérez-Torres, A., Escalona-Montaña, A. R., & Gutiérrez-Kobeh, L. (2020). Differential regulation of L-arginine metabolism through arginase 1 during infection with *Leishmania mexicana* isolates obtained from patients with localized and diffuse cutaneous leishmaniasis. *Infection and immunity*, 88(7), 10.1128/iai.00963–00919.
- Wulandari, S., Sholikah, T. A., & Jusup, S. A. (2026). The baseline levels of receptors and cytokines in phorbol 12-myristate 13-acetate-induced THP-1 monocyte to macrophage cells. *Indonesian Journal of Medical Laboratory Science and Technology*, 8(1), 24–32.
- Zuniga, T. M. (2018). LPS-Induced iNOS mRNA and the Pro-Apoptotic Signaling Pathway in Leukocytes of Fit and Unfit Males.

### **Statements & Declarations**

The authors declare that no funds, grants, or other support were received during the preparation of this manuscript

### **Competing Interests**

The authors have no relevant financial or non-financial interests to disclose.

### **Author Contributions:**

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by *Khawla Adnan Baqir*, and *Sarmad Nory Gany*. The first draft of the manuscript was written by Khawla Adnan Baqir and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

### **Data Availability:**

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

### **Ethics approval:**

No ethical approval is required.