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RESEARCH ARTICLE

# Potent antileishmanial and synergic effects of phellandrene through its immunomodulation, modulating oxidant/antioxidant, and apoptotic induction

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**ABSTRACT**

The primary pharmaceutical treatments for the cutaneous leishmaniasis (CL) are linked to a range of negative complications. The development of innovative pharmacological agents aimed at enhancing cellular immune responses could denote a promising therapeutic approach for CL therapy. Here, the present study investigated the impact of phellandrene (PR), a cyclic monoterpene found in various plant species, on *Leishmania tropica*, focusing on its antileishmanial properties, immunomodulatory effects, antioxidant activity, and capacity to induce apoptosis. The antileishmaial and synergistic properties effects of PR alone and in conjunction with glucantime (GCT) on *L. tropica* promastigote and amastigote forms were investigated. As well, the influence of PR on the immunomodulatory-associated genes, antioxidant-associated genes, plasma membrane integrity, ROS generation, apoptosis induction, and nitric oxide (NO) production was assessed. We found that PR principally in conjunction with GCT notably reduced by the number of promastigote and amastigote forms within macrophages a dose-dependent reduction ($p<0.001$). We found a significant upregulation in the expression of the *iNOS*, interferon gamma *(IFN-γ)*, and tumor necrosis factor *(TNF-α)* genes in infected macrophages subsequent to treatment with RP, particularly in conjunction with GCT. Conversely, there was a notable downregulation in the expression of interleukin 10 (*IL-10),* superoxide dismutase *(SOD),* and catalase *(CAT)* genes; whereas, results in a substantial rise in NO release in macrophage cells ($p<0.001$). PR, GCT, PR+GCT resulted in a dose-dependent enhancement of caspase-3 activity, increase in plasma membrane integrity, and reactive oxygen species (ROS) production ($p<0.001$). The findings indicate that the PR mainly along with GCT has a substantial effect on the inhibition and elimination of *Leishmania* parasites in controlled laboratory environments. Although certain cellular mechanisms of action have been recognized, including immune modulation cellular immunity response, the induction of apoptosis, ROS and NO production, reducing the antioxidant activity, and affecting membrane integrity in response to *Leishmania*, additional research is required to interpret its effectiveness in both animal models and human participants.

**Keywords:** monoterpenes; *Leishmania*; apoptosis; cellular immunity; oxidative stress.

## INTRODUCTION

Leishmaniasis is a prevalent disease that impacts both human and animal populations on a global scale (Monzote, 2009). This disease is capable of transmission between humans and various animal species, via the bites of sandflies from the *Phlebotomus* and *Lutzomyia* genera (Monzote, 2009). Cutaneous leishmaniasis (CL) represents one of the most frequently encountered manifestations of the disease, with a substantial incidence observed in nations such as Saudi Arabia (Torres-Guerrero *et al*., 2017). The principal pharmacological interventions for the condition include pentavalent antimony compounds, such as meglumine antimoniate and pentostam. Furthermore, treatment regimens may also incorporate additional agents, including amphotericin B and paromomycin (Pradhan *et al*., 2022). Meglumine antimoniate, referred to as glucantime (GCT), is a frequently prescribed pharmacological agent. However, its use is often associated with common adverse effects, especially in patients with hepatic and renal impairments. Additionally, the high costs and the emergence of resistance to GCT have stimulated increased research efforts aimed at identifying alternative therapeutic approaches (Oliveira *et al*., 2011; Albalawi *et al*., 2021).

Currently, herbs and their derivatives are acknowledged as important sources of a wide variety of valuable therapeutic compounds. This recognition is due to their high accessibility, cost-effectiveness, low toxicity, and considerable efficacy (Ghodsian *et al*., 2020). Monoterpenes, which consist of hydrocarbon structures, constitute the largest group of plant secondary metabolites and are commonly found in essential oils (Koziol *et al*., 2014). Phellandrene (PR, $C_{10}H_6$), a cyclic monoterpene, is found in various plant species, especially within the genera *Curcuma, Nigella, Salvia,* and *Piper* (Thangaleela *et al*., 2022). Investigations in the biological sciences have demonstrated that Phellandrene exhibits a wide range of pharmacological benefits, including the suppression of

inflammation, the mitigation of oxidative stress, the inhibition of cancerous growths and tumors, and antimicrobial properties (Radice *et al*., 2022). A significant amount of research has demonstrated the efficacy of various herbs (*Artemisia* spp., *Calendula* spp., *Zataria* spp., *Allium* spp., *Myrtus* spp., and *Mentha* spp.) and their derivatives (e.g., monoterpenes, phenols, and flavonoids), in the treatment of CL (Shooraj *et al*., 2022). Nevertheless, the conclusions drawn from these studies are limited by several factors, including an insufficient comprehension of the fundamental mechanisms at play and issues regarding potential toxicity (Parvizi *et al*., 2020).

Research indicates that inflammatory cytokines play a significant role in influencing both susceptibility and resistance, as well as in the immunopathogenesis of leishmaniasis (Mirzaei *et al*., 2021). Alterations in the equilibrium of cytokine levels can influence clinical signs and may function as potential biomarkers for these outcomes (Saha *et al*., 2022). Specifically, inflammatory cytokines linked to T helper 1 (Th1) cells, such as interferon-$\gamma$, tumor necrosis factor-$\alpha$, and interleukin-12 (IL-12), are essential for the development of immunity against CL (Saha *et al*., 2022). In contrast, Th2 cytokines, including IL-10 and IL-13, facilitate the persistence of the parasite by inhibiting the Th1 immune response (Maspi *et al*., 2016). Accordingly, the development of innovative pharmacological agents aimed at enhancing cellular immune responses may represent a a potentially effective therapeutic strategy for the management of the CL (Maspi *et al*., 2016). The aim of this research was to investigate the impact of PR on *L. tropica,* focusing on its antileishmanial properties, immunomodulatory effects, and capacity to induce apoptosis.

## MATERIALS AND METHODS

**Chemicals**

PR, exhibiting a purity of 99%, MTT powder, along with PY with a minimum purity of 98%, Caspase-3 Activity kit, and Nitrite Assay Kit were procured from Sigma-Aldrich, Germany.

**Parasite and cells**

*L. tropica* (MHOM/IQ/1992/MREC3) and THP-1 human monocytic cells (ATCC) were kept in RPMI1640 medium and DMEM (Gibco, USA), developed with 10% FBS and pen-strep (100 mL / IU) at 23±1°C and at 37°C with 5% CO2, respectively.

**Anti- *L. tropica* promastigotes effects of PR**

This assay was performed as triplicate based on the method reported by Saedi Dezaki *et al*. (2016). In summary, promastigotes (0.1 mL at a concentration of 1000000 per mL) were allocated into a 96-well plate. Following this, varying concentrations of PR (0.1 mL, ranging from 6.25 to 100 µg/mL) were added and incubated at 24°C for a duration of two days. Afterward, 20 µL of thiazolyl blue tetrazolium bromide (MTT) at 500 µg/mL (Sigma-Aldrich, USA) was added and incubated for an additional four hours under the same conditions. Following this incubation, dimethylsulfoxide was added, and the OD was recorded at 570 nm using a microplate-ELISA reader (STAT-FAX-3200, USA).

**Evaluation of synergistic effects**

The synergistic potential of CP in conjugation with PY, we computed the fractional inhibitory concentration index (FICI) utilizing the subsequent formula (Odds, 2003):

$$\frac{\text{IC50 in conjugation}}{\text{(IC50 alone)}}$$

Synergistic effects: $\leq 0.5$
Additive effects: 0.5-1
Antagonist effects: 1< FICI<4

**Effect of PR on the membrane integrity**

In summary, promastigotes (1000000 per mL) were subjected various concentrations of PR alone and in conjunction in GCT to in 96-well plates for 48; whereas, the assay was performed as triplicate. Later, 0.1 mL of to Sytox Green (Invitrogen, USA) were introduced to the wells and left for 7h. The fluorescence emitted at 480-522 nm was then quantified hourly over a four-hour period utilizing a microplate reader.

**Effect of PR on amastigotes within macrophages**

Firstly, 100.000 cells in a volume of 0.1 mL, were sited into a 24-well plate; whereas, the bottom of the containers was equipped with coverslips and was kept in a controlled state at 37°C and 5% CO2. Following this, 1,000,000 promastigotes in a volume of 0.1 mL (stationary phase) were introduced to the macrophage cells under identical conditions for one day. Subsequent to the introduction of varying concentrations of PR (5-100 µg/mL), GCT (5-100 µg/mL), and GCT (5-100 µg/mL) in combination with PR (at IC50 concentration) the resulting combination was subjected to an additional incubation period of two days. In the concluding phase of the experiment, the slides were subjected to Giemsa staining (Merck, Germany), and the effectiveness of the drug was evaluated by analyzing 100 macrophages using a light microscope (Ezatpour *et al*., 2015; Mahmoudvand *et al*., 2017). The assay was performed as triplicate and half-maximal inhibitory concentration (IC50) was determined by Probit test.

**Effect of PR on the immunomodulatory-associated genes in macrophages**

The gene expression of T Lymphocytes- associated genes nitric oxide synthase (iNOs), interferon gamma (IFN-$\gamma$), and tumor necrosis factor (TNF-$\alpha$), and interleukin 10 (IL-10), in THP-1 cells infected and subsequently treated with CP were analyzed using quantitative reverse transcription polymerase chain reaction (qRT-PCR). Firstly, the total RNA extraction was performed according to the RNA extraction protocol (Plus-RNX Cinagene, Iran). To determine the quantity and quality of RNA extraction, optical absorbance measurement was performed. One microgram of extracted RNA was utilized for the synthesis of complementary DNA (cDNA) employing the Qiagen cDNA synthesis kit. Relative quantification reactions were performed in which the target *iNOs*, *TNF-$\alpha$, IFN-$\gamma$,* and *IL-10* genes (Table 1) were compared to the expression of a housekeeping gene ($\beta$-actin) using the StepOnePlus™ RT-PCR (Thermo Fisher Scientific, USA) and Qiagen SYBR Green RT-PCR kit. The procedure commenced with an initial denaturation phase conducted at a temperature of 95°C for 10 min. Afterwards, the procedure involved 40 cycles, with each cycle comprises a denaturation phase at 95°C for 10 seconds, followed by an annealing and extension phase at 60°C for 30 seconds. Ultimately, the comparative Ct method ($2^{-\Delta\Delta CT}$) was employed to investigate relative alterations in gene expression levels (Mahmoudvand *et al*., 2024).

**Assessment of nitric oxide (NO) creation**

The assay was conducted utilizing the Nitrite Assay Kit in accordance with the manufacturer's instructions. In summary, a concentration of 100,000 macrophage cells per milliliter was treated with PR, LPS-IFN-$\gamma$ (serving as a positive control), and normal saline (serving as a negative control) for a period of 48 hours in a 96-well plate format. Following this incubation, 20 µL of the supernatant was mixed with 80 µL of buffer, along with Griess reagents A and B. The optical density (OD) of the resultant solution was subsequently measured at 540 nm using an ELISA reader.

**Assessment of apoptosis induced by Caspase-3 like enzyme activity**

The evaluation of apoptotic activity was conducted using the Caspase-3 Activity Assay Kit, following the manufacturer's instructions. After a 48-hour incubation period of infected

macrophages with PR, the samples were subjected to centrifugation at 4°C. The resulting pelleted were then lysed and centrifuged again at 15,000 rpm for 15 minutes. Following this, a volume of 5 µL from the upper phase was combined with a buffer and the caspase substrate (pNA-DEVD-Ac), and the resulting mixture was incubated for 2 hours at 37°C. The optical density of the final solution was measured at a wavelength of 405 nm using an ELISA reader (Cheraghipour *et al*., 2024).

**Impact of CP on reactive oxygen species (ROS)**

The assay utilizing the dye 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA) was employed to quantify the extent of intracellular amastigote oxidation through the release of ROS in macrophages that had been infected. Infected mice that received treatment with PR were subsequently placed in 1.5 mL microcentrifuge tubes at a specified cell density per well and incubated at 37°C with 5% CO2 for a duration of three days. The measurement of ROS levels was conducted in accordance with the manufacturer's instructions (Sigma-Aldrich, USA), which involved the application of the commonly utilized fluorogenic probe DCFH-DA. The fluorescence emitted at 488-525 nm was then quantified hourly over a four-hour period utilizing a microplate reader (Yu *et al*., 2021).

**Effect of PR on the antioxidant-associated genes**

In order to assess alterations in the relative expression of genes and the average production of antioxidant genes, superoxide dismutase (SOD) and catalase (CAT) in *L. tropica* intra-macrophage amastigotes. qRT-PCR techniques were employed in which the target *SOD*, and *CAT* genes (Table 1) were compared to the expression of a housekeeping gene (β-actin) using the StepOnePlus™ RT-PCR (Thermo Fisher Scientific, USA) and Qiagen SYBR Green RT-PCR kit based on the details and temperature conditions described above.

**Cell cytotoxicity of CP on human normal cells**

This study employs the MTT assay, adhering to the protocols established in anti- *L. tropica* promastigotes effects of PR (Mahmoudvand *et al*., 2016); whereas, THP-1 cells, at a concentration of 100000 cells/mL, were subjected to treatments with either PR alone, GCT alone, or a combination of PR and GCT in a 96-well plate for a duration of 72 hours. Subsequently, the absorbance was measured at 590 nm using an ELISA plate reader. The 50% cytotoxicity concentrations ($CC_{50}$) was determined by Probit test. The selectivity index (SI) were subsequently calculated as (Ghasemian Yadegari *et al*., 2023):

$$\frac{\text{CC50 of normal cells}}{\text{IC50 of intracellular parasites}}$$

**Data analysis**

All *in vitro* tests in this study were carried out as triplicate. The analysis of variance (ANOVA), along with Tukey's test and other *post-hoc* analyses, was utilized to conduct a more in-depth examination of the data in SPSS version 24.0 software, with a significance level established at P<0.05.

## RESULTS

**Anti- *L. tropica* promastigotes effects**

The viability of promastigotes was notably reduced by PR (p<0.001), as illustrated in Figure 1A. The IC50 for PR, GCT, and the combination of PR + GCT were determined to be 61.2, 89.6, and 29.4 µg/mL, respectively. It is noteworthy that the combination of PR and GCT demonstrated the most significant anti-amastigote activity. (p<0.05).

**Table 1.** The sequence of the used primers for real-time PCR

| Amplicon | Sequence (5'–3') |
|---|---|
| IFN-γ | F: TGCCGGAAGGCGGCTCATTC<br>R: CGCAGTGCGTTGCGCATACC |
| TNF-α | F: CTGGGGCCTACAGCTTTGAT<br>R: GGCTCCGTGTCTCAAGGAAG |
| IL-10 | F: AAGGCAGTGGAGCAGGTGAA<br>R: CCAGCAGACTCAATACACAC |
| iNOs | F: TGCCGGAAGGCGGCTCATTC<br>R: CGCAGTGCGTTGCGCATACC |
| SOD | F: TATGGGGACAATACACAAGGCT<br>R: CGGGCCACCATGTTTCTTAGA |
| CAT | F: GGAGGCGGGAACCCAATAG<br>R: GTGTGCCATCTCGTCAGTGAA |
| β-actin | F: GTGACGTTGACATCCGTAAAGA<br>R: GCCGGACTCATCGTACTCC |

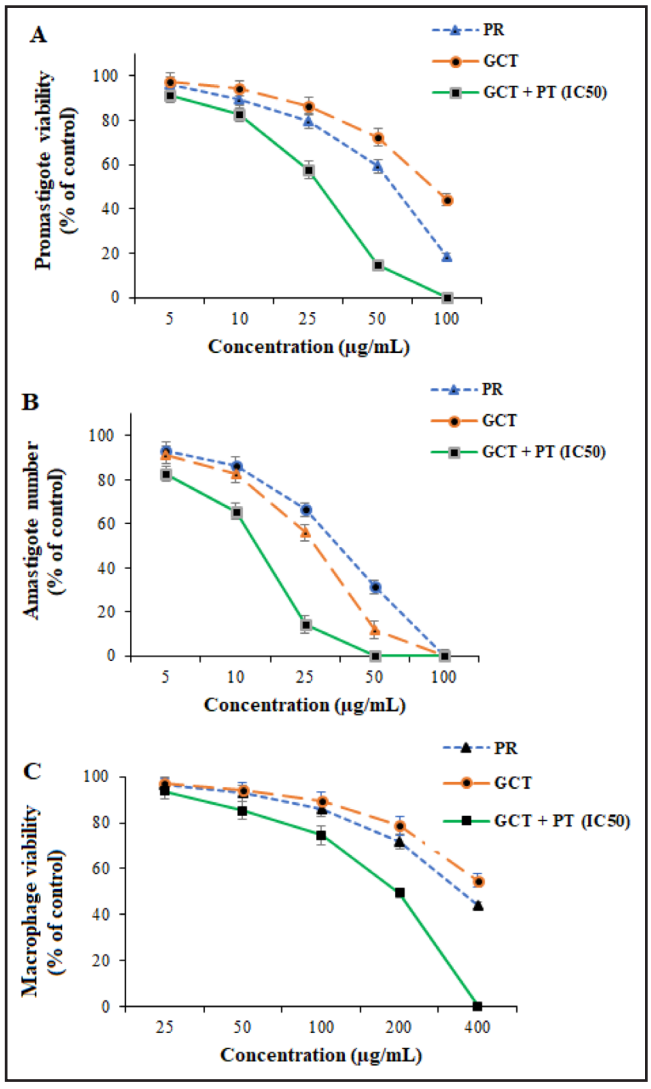


**Figure 1.** The *in vitro* anti-leishmanial and cytotoxicity effects of phellandrene (PR) and glucantime (GCT), and the combination of them against promastigotes (A), amastigotes (B), and macrophage cells (C). The results are presented as Mean ± SD, and the experiments were conducted in triplicate (N=3).

**Synergic effects PR and GCT**

The calculated FICI values for PR and GTC were 0.48 and 0.32, respectively, suggesting the presence of synergistic effects when PR is administered in conjunction with GCT, as presented in Table 2.

**Effect on amastigote within macrophages**

According to the results obtained from the macrophage model, PR demonstrated a dose-dependent reduction in the viability of amastigote within macrophages (Figure 1B). The IC50 for PR, GCT, and the combination of PR + GCT were determined to be 34.4, 28.4, and 14.6 µg/mL, respectively ($p > 0.05$) (Table 2).

**Cytotoxic effects and SI on macrophage cells**

Figure 1C presents the cytotoxic effects of PR, GCT, and the combination of PR + GCT on THP-1 cells, as evaluated through the MTT assay. The results demonstrate that PR, GCT, and the combination of PR + GCT reduced cell viability with the $CC_{50}$ values of 355.1, 475.1, and 197.4 µg/mL for PR, GCT, and the combination of PR + GCT, respectively. Furthermore, the SI value for PR, GCT, and the combination of PR + GCT were considered to be 10.3, 16.1, and 13.5, respectively (Table 2).

**Effect of PR on the membrane integrity**

The findings indicate that the administration of PR to promastigotes principally along with GCT leads to a dosage-dependent enhancement of plasma membrane integrity ($p<0.001$), as demonstrated by the observed increase in fluorescence (Figure 2). The most pronounced effects on plasma membrane integrity in promastigotes were recorded 21.4%, 49.3%, and 60.1%, after exposure to PR+GCT at 1/3 IC50, ½ IC50, and IC50, respectively.

**Caspase-3-like activity of extract-treated parasites**

The findings demonstrated that the exposure of infected macrophages to PR, GCT, PR+GCT resulted in a dose-dependent enhancement of caspase-3 activity versus to cells treated with normal saline (Figure 3). The highest levels of caspase-3-like enzyme activity were observed at 8.7%, 10.3%, 18.8%, and 26.3% following treatment with PR+GCT at ¼ IC50, 1/3 IC50, ½ IC50, and IC50 concentrations, respectively.

**Effect on NO release**

Griess assay showed a substantial rise in NO release in macrophage cells following the exposure of PR, with statistical significance ($P<0.001$) observed particularly in conjunction with GCT at 1/3 IC50 (8.6 µM), ½ IC50 (22.1 µM) and IC50 (30.8 µM) concentrations, in comparison to the cells treated with normal saline (Figure 4).

**Table 2.** A comparative analysis of the 50% inhibitory concentration (IC50), the fractional inhibitory concentration index (FICI), the 50% cytotoxic concentration (CC50), and the selectivity index (SI) for phellandrene (PR) and glucantime (GCT), and the combination of them. *** P< 0.001 significant difference compared with GCT by *post hoc* analysis

| Drug | IC50 for promastigote (µg/mL) | FICI | IC50 for amastigote (µg/mL) | CC50 for THP-1 cells (µg/mL) | SI |
|---|---|---|---|---|---|
| PR | 61.2±3.36 | 0.48 | 34.4±3.78 | 355.1±7.63 | 10.3 |
| GCT | 89.6±5.21 | 0.32 | 28.4±1.66 | 457.1±9.26 | 16.1 |
| GCT+PR | 29.4±2.66*** | – | 14.6±1.15*** | 197.4±4.16*** | 13.5 |

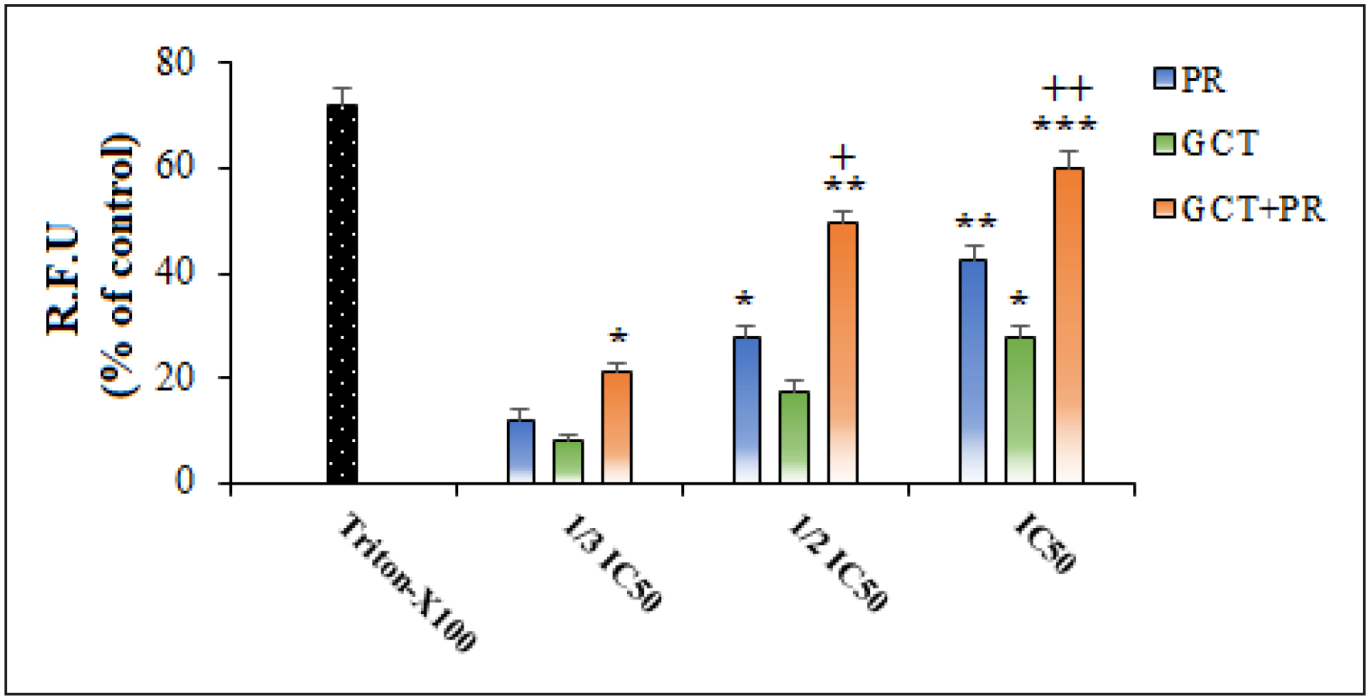


**Figure. 2.** The level of the membrane integrity in *Leishmania* promastigotes subjected to phellandrene (PR) and glucantime (GCT), and the combination of them. The results are presented as Mean ± Standard Deviation (SD). The experiment was conducted in triplicate (N=3). Statistical significance was indicated with * $p<0.05$, ** $p< 0.01$, and *** $p<0.01$ versus normal saline; + $p<0.05$; and ++ $p<0.01$ versus GCT by *post hoc* analysis.

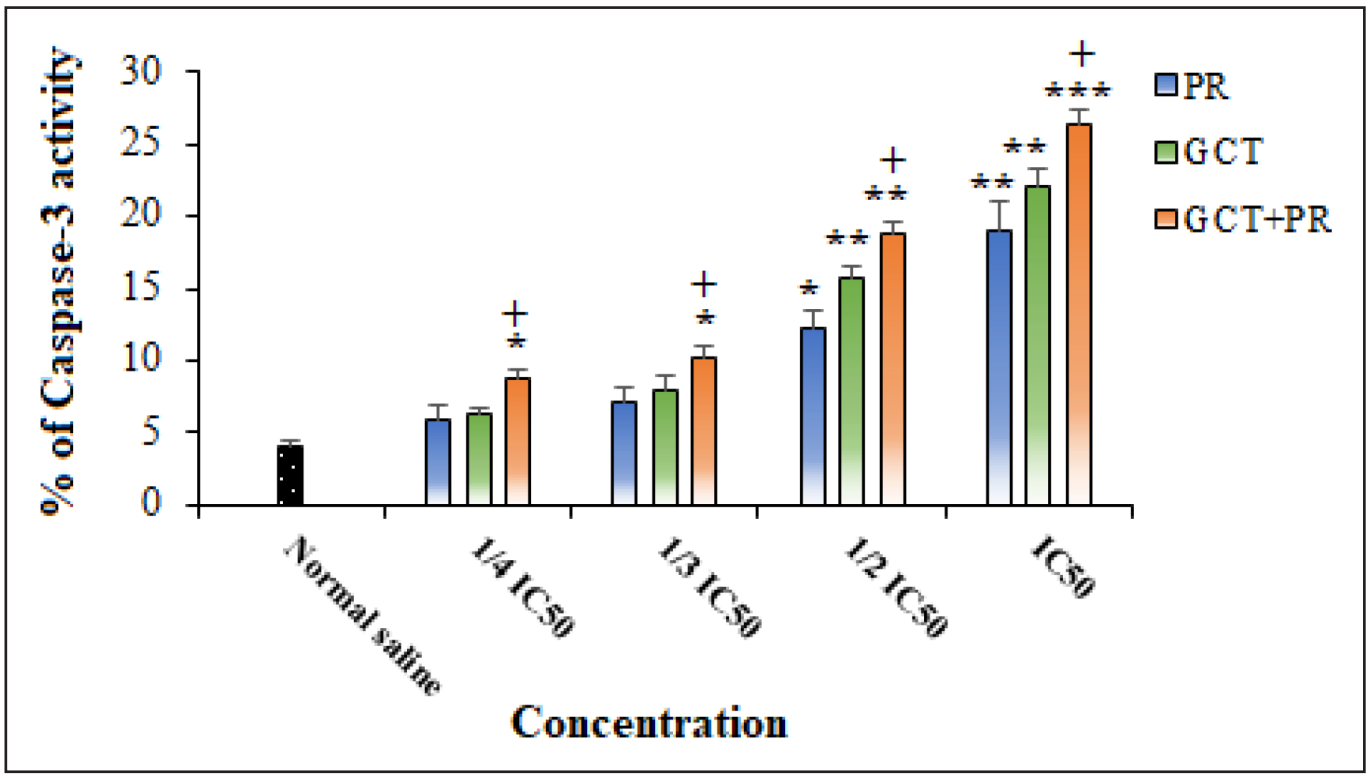


**Figure 3.** The activity level of the caspase-3 enzyme in *Leishmania* infected macrophages subjected to phellandrene (PR) and glucantime (GCT), and the combination of them. The results are presented as Mean ± Standard Deviation (SD). The experiment was conducted in triplicate (N=3). Statistical significance was indicated with * p<0.05, ** p< 0.01, and *** p<0.01 versus normal saline; + p<0.05 versus GCT by *post hoc* analysis.

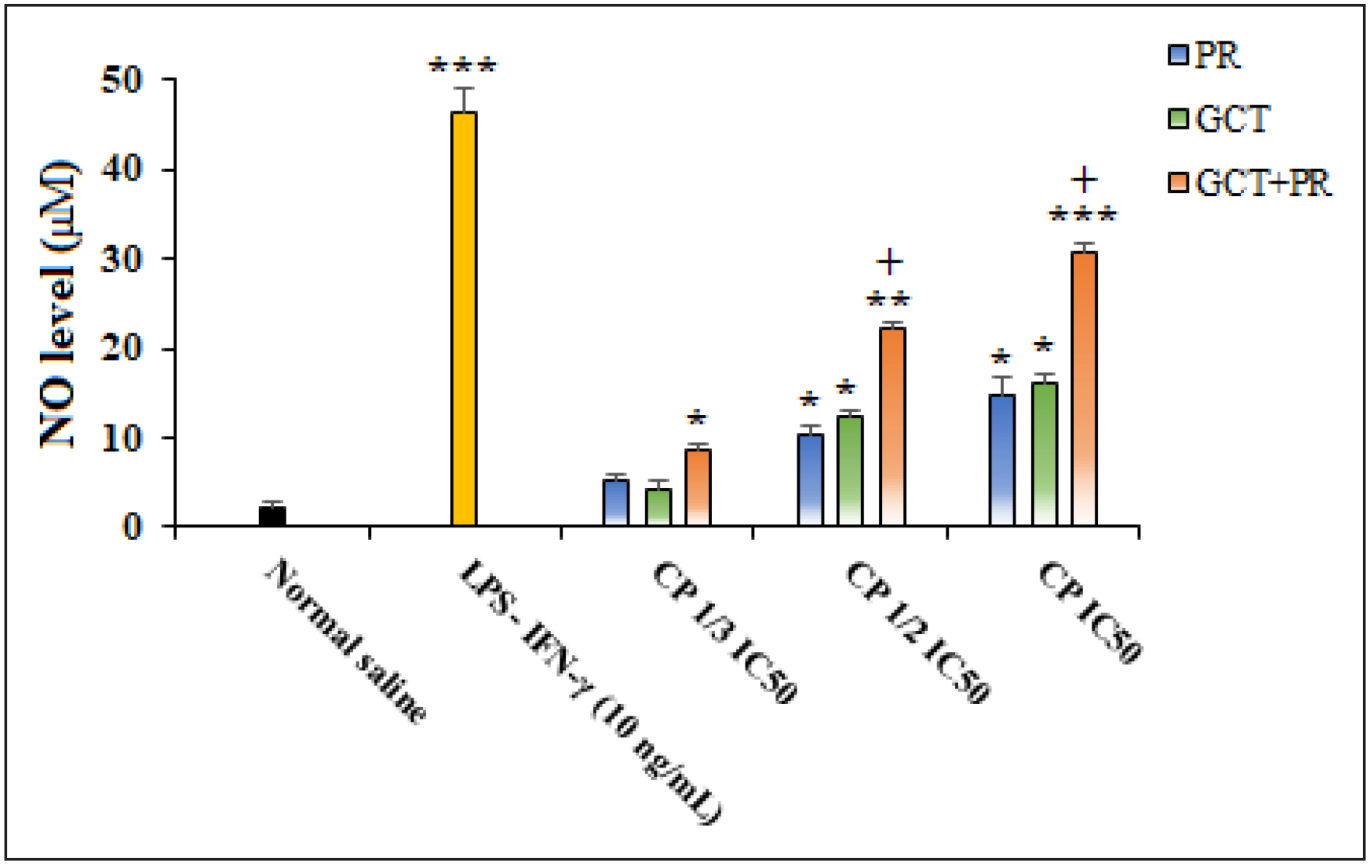


**Figure 4.** The production of nitric oxide (no) in macrophages infected with *Leishmania tropica* following treatment with phellandrene (PR) and glucantime (GCT), and the combination of them. The results are presented as Mean ± SD, with the experiments conducted in triplicate (N=3). Statistical significance is indicated as * p<0.05, ** p< 0.01, and *** p<0.01 versus normal saline; + p<0.05 versus GCT by *post hoc* analysis.

**Effect on the ROS generation**

The findings indicated that PR principally combined with GCT pointedly augmented fluorescent intensity and afterward ROS production is dependent on the dosage (p<0.001) versus the normal saline (Figure 5). The highest fluorescent intensity was observed 19.3, 30.1, 38.8, and 46.2 a.u. after exposure of infected macrophages to PR+GCT at ¼ IC50, 1/3 IC50, ½ IC50, and IC50 concentrations, respectively.

**Effect of PR on the antioxidant-associated genes**

Figure 6 demonstrates a notable increase in the expression levels of the SOD and CAT genes in untreated intra-macrophage amastigotes (p<0.001). Conversely, there was a notable downregulation in the expression of *SOD* and *CAT* genes in intra-macrophage amastigotes treated with PR (P<0.001). Notably, the combination treatment of PR and GCT exhibited the most reduction in the expression levels for the *SOD* (0.76-fold change) and CAT (0.81-fold change) genes in comparison with PR and GCT alone (p<0.001).

**Effect on the immunomodulatory-associated genes in macrophages**

Figure 7 illustrates a significant upregulation in the expression of the *iNOS, IFN-γ,* and *TNF-α* genes in infected macrophages subsequent to treatment with RP, particularly in conjunction with GCT. Conversely, there was a notable downregulation in the expression of *IL-10* gene (Figure 7). Notably, the combination treatment of PR and GCT exhibited the most pronounced expression levels for the *iNOS* (6.21-fold change), *IFN-γ* (4.89-fold change), and *TNF-α* (5.12-fold change) genes, alongside the most significant downregulation of *IL-10* (1.02-fold change), with statistical significance established at p<0.001.

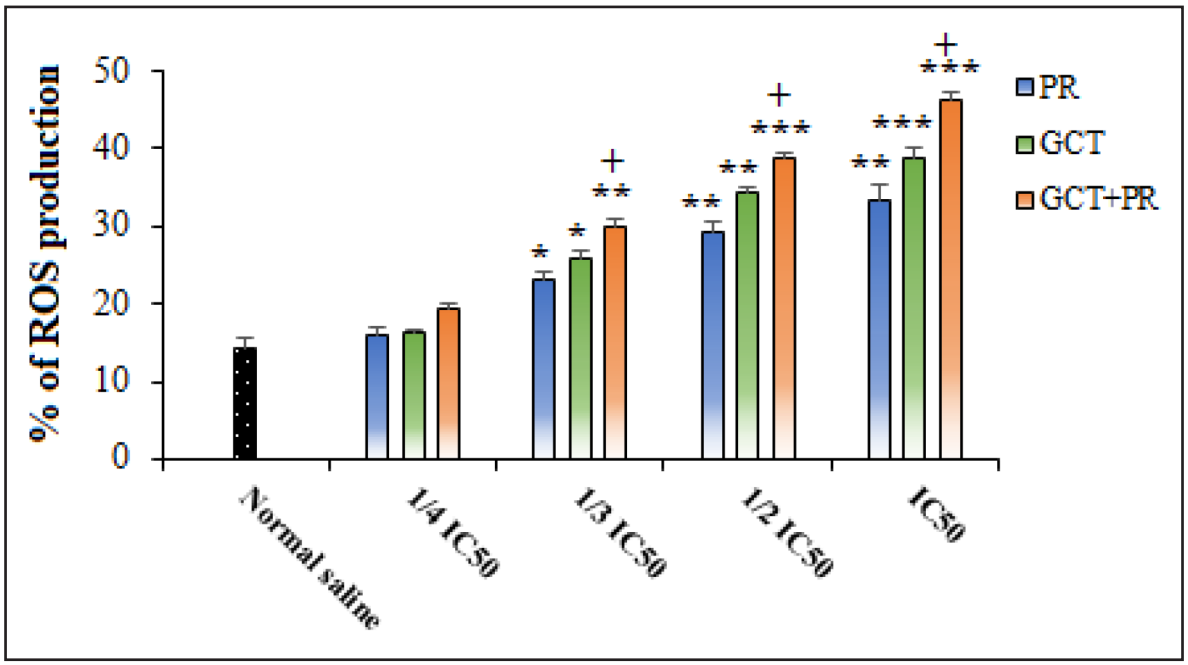


**Figure 5.** The production of reactive oxygen species (ROS) in macrophages infected with *Leishmania tropica* following treatment with phellandrene (PR) and glucantime (GCT), and the combination of them. The results are presented as Mean ± SD, with the experiments conducted in triplicate (N=3). Statistical significance is indicated as * p<0.05, ** p< 0.01, and *** p<0.01 versus normal saline; + p<0.05 versus GCT by *post hoc* analysis.

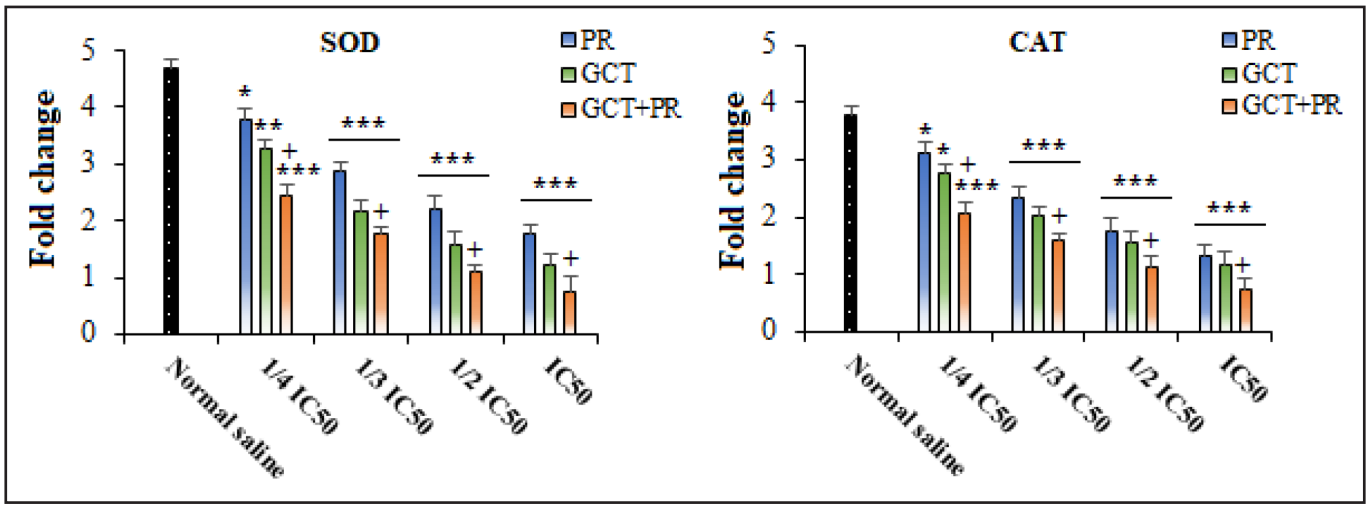


**Figure 6.** The expression levels of superoxide dismutase (SOD) and catalase (CAT) in *Leishmania tropica* intracellular amastigotes with following treatment with phellandrene (PR) and glucantime (GCT), and the combination of them. The results are presented as Mean ± Standard Deviation (SD). The experiments were conducted in triplicate (N=3). Statistical significance was determined with * p<0.05, ** p< 0.01, and *** p<0.01 versus normal saline; + p<0.05 versus GCT by *post hoc* analysis.

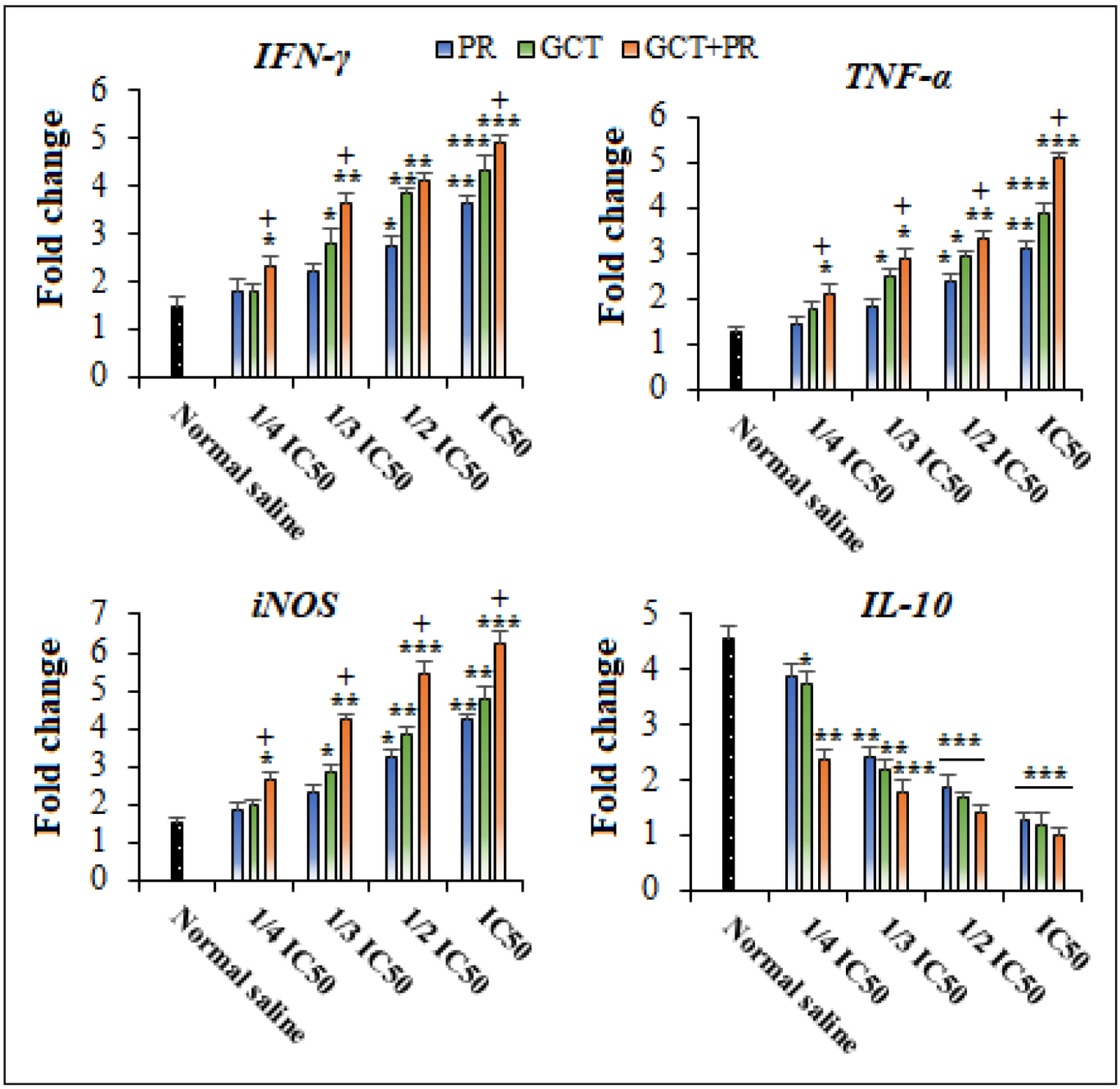


**Figure 7.** The expression levels of Interferon-gamma (IFN-γ), Interleukin 10 (IL-12), and Tumor Necrosis Factor alpha (TNF-α), and nitric oxide synthase (iNOS) in *Leishmania tropica* macrophages infected with following treatment with phellandrene (PR) and glucantime (GCT), and the combination of them. The results are presented as Mean ± Standard Deviation (SD). The experiments were conducted in triplicate (N=3). Statistical significance was determined with * p<0.05, ** p< 0.01, and *** p<0.01 versus normal saline; + p<0.05 versus GCT by *post hoc* analysis.

## DISCUSSION

Currently, compounds derived from natural sources are recognized for their considerable potential as candidates for antiparasitic pharmacotherapy (Ndjonka *et al*., 2013). Additionally, phytomedicines are increasingly acknowledged as cost-effective and safe alternatives to conventional treatment methods (Ndjonka *et al*., 2013). At present, the standard therapeutic strategy for the prevention and management of CL involves the use of chemical agents, which are often linked to significant adverse side effects (Ghodsian *et al*., 2020). Despite notable progress in pharmacological research and safety evaluations, there remains a considerable gap in the availability of an optimal and effective therapeutic intervention for CL. The current study aims to investigate the impact of PR on *L. tropica*, focusing on its antileishmanial properties, immunomodulatory effects, and capacity to induce apoptosis.

The current results showed that PR principally in conjunction with GCT notably reduced by the viability *of L. tropica* promastigote and amastigote forms within macrophages a dose-dependent reduction. The results also exhibited that the obtained FICI values of lower 0.5 for PR and GCT, indicating the presence of synergistic effects when PR is used in conjunction with GCT. Prior research has demonstrated that naturally occurring small molecules can serve as effective delivery systems, thereby improving bioavailability, reducing toxicity, augmenting drug concentration, and prolonging systemic circulation. These characteristics can substantially enhance the efficacy of conventional pharmaceuticals (Fu & Yang, 2023).

Recent researches have provided evidence for the antimicrobial properties of PR and herbal essential oils rich in PR against a wide range of microorganisms, including various bacterial strains such as *Staphylococcus* spp., *Streptococcus* spp., and *Pseudomonas* spp., as well as fungal species like Candida spp., and *Aspergillus* spp. (Thangaleela *et al*., 2022). Additionally, these substances have demonstrated efficacy against viral pathogens, including *Bacillus* phage CP51 and Herpes simplex virus (Radice *et al*., 2022). By term of antiparasitic effects of PR, studies showed that there are some PR-rich essential oils that displayed potent *in vitro* and *in vivo* antiparasitic effects against several *Leishmania* spp (Radice *et al*., 2022). For example, Mahmoudvand *et al*. (2022) reported that PR-essential oil of *Ferula macrecolea* showed effective leishmanicidal effects with IC50 of 27.6 and 42.3 µg/mL on promastigote and amastigotes of *L. tropica*. Almohammed *et al*. (2022) reported that PR-rich essential oil of *Elettaria cardamomum* exhibited the promising antiparasitic efficacy at a concentration of 200 µg/ml, whereas, the treatment resulted in the complete destruction of 100% of the *Echinococcus granulosus* protoscolices following a 10-minute incubation period. de Lima Nunes *et al*. (2021) reported that PR-rich essential oil of *Eugenia piauhiensis* had favorable effects against promastigote and amastigote forms of *L. amazonensis* with IC50 values of 6.43 and 4.59 µg/mL, respectively. The difference in the results can be attributed to type of parasite, type of plant, the used method, the used concentrations.

While the special antimicrobial mechanisms of PR are not fully explained, researches have suggested that monoterpene compounds demonstrate antimicrobial properties by altering the lipid composition of microbial plasma membranes (Trombetta *et al*., 2005). This alteration results in modifications to membrane permeability, which in turn causes the leakage of intracellular components and changes in pH and ATP levels. Furthermore, these compounds impact the structural integrity of lipids in bacterial membranes, interfere with DNA, and disrupt cellular motility. They also facilitate the production of reactive oxygen species (ROS), initiate apoptotic processes, and affect electron localization (Trombetta *et al*., 2005; Marchese *et al*., 2017).

In recent years, the induction of apoptosis has emerged as a promising antimicrobial strategy associated with various drugs currently under investigation (Wanderley & Barcinski, 2010). Caspases are integral to this process, with Caspase-3 being recognized as the principal caspase that activates death proteases, thereby initiating cell death (Basmaciyan & Casanova, 2019). The findings demonstrated that the exposure of infected macrophages to PR, GCT, PR+GCT resulted in a dose-dependent enhancement of caspase-3 activity versus to cells treated with normal saline. In a related study, Susanto *et al*. (2024) demonstrated that PR promotes the apoptosis of HT-29 cells that is induced by 5-fluorouracil through the modulation of the mitochondria-dependent pathway. Consequently, it is plausible to suggest that the administration of PR may effectively induce apoptosis, thereby aiding in the elimination of promastigotes.

Inflammatory cytokines substantially influence both susceptibility and resistance to leishmaniasis, as well as its immunopathogenesis; whereas, they may serve as potential biomarkers for CL outcomes (Saha *et al*., 2022). Furthermore, the term "NO" is extensively acknowledged as a crucial component of the innate immune response, playing a significant role in the regulation and elimination of intracellular pathogens, including *Leishmania* spp. (Saha *et al*., 2022). Therefore, the development of novel pharmacological agents designed to enhance cellular immune responses may offer a promising therapeutic strategy for the treatment of CL. The results found a significant upregulation in the expression of the *iNOS, IFN-γ,* and *TNF-α* genes in infected macrophages subsequent to treatment with RP, particularly in conjunction with GCT. Conversely, there was a notable downregulation in the expression of *IL-10* gene; whereas, results in a substantial rise in NO release in macrophage cells. It has been proposed that the increased expression of cytokines associated with T lymphocytes, along with the production of NO by PR, may represent a potential mechanism for the regulation of *L. tropica* parasites. Recent investigations underscore that the principal mechanisms for the eradication of pathogenic microorganisms involve the disruption of plasma membrane integrity and the production of ROS (Hohenberger *et al*., 2011; Memar *et al*., 2018). Researches have demonstrated that elevated levels of ROS serve as a lethal mechanism employed by phagocytic cells to inflict damage on essential biomolecules, including proteins, lipids, and DNA, ultimately resulting in apoptosis (Memar *et al*., 2018). Furthermore, it has been established that leishmanial agents can generate ROS as a consequence of cellular processes and drug absorption. Numerous chemotherapeutic agents utilized in the treatment of Leishmania species and cancer exert their therapeutic effects through the induction of ROS production (Memar *et al*., 2018). *Leishmania* parasites stimulate the production of various types of interferons by infected macrophages, which subsequently increases the expression of SOD and CAT enzymes. These enzymes function as effective antioxidants, facilitating the activation of macrophages to generate proteolytic enzymes and ROS intermediates that play a role in the elimination of the parasite. SOD and CAT mitigate the detrimental effects of ROS in the environment by catalyzing the dismutation of superoxide radicals into hydrogen peroxide, water, and molecular oxygen (Hohenberger *et al*., 2011). In this research, the results showed that the administration of PR principally along with GCT to *Leishmania* parasites led to an increase in plasma membrane integrity and ROS production, which was contingent upon the dosage administered; whereas, there was a notable downregulation in the expression of SOD and CAT genes in intra-macrophage amastigotes treated with PR ($P<0.001$). These findings are consistent with previously established antimicrobial mechanisms associated with monoterpene compounds. It is proposed that PR may exert its antileishmanial effects primarily by enhancing plasma membrane permeability, promoting ROS generation and reducing the antioxidant activity. In the examination of the cytotoxic effects of PR, the results indicate that PR, GCT, and PR+GCT resulted in a reduction of cell viability. Furthermore, the results demonstrated that the SI values for PR, GCT, and PR+GCT were greater than 10, which implies their relative safety for macrophages and their specificity towards the parasite, corroborating the findings of Weniger *et al*. (2001). Previous study has documented the cytotoxic effects of PR on colorectal adenocarcinoma cancer cell lines with IC50 of 250 µM (Susanto *et al*., 2024).

The recent research has highlighted the potential antileishmania effects of PR. However, it is essential to further explore the pharmacodynamic and pharmacokinetic effects are required to evaluate the compound's absorption, bioavailability, and metabolic pathways. To validate its therapeutic application, it is strongly recommended that clinical studies be conducted, encompassing assessments of toxicity and therapeutic efficacy to substantiate the therapeutic potential of this molecule.

## CONCLUSION

The findings indicate that the PR mainly along with GCT has a substantial effect on the inhibition and elimination of *Leishmania* parasites in controlled laboratory environments. Although certain cellular mechanisms of action have been identified, including immune modulation cellular immunity response, the induction of apoptosis, ROS and NO production, reducing the antioxidant activity, and affecting membrane integrity in response to *Leishmania* parasites, additional research is required to interpret its effectiveness in both animal models and human participants.

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We declare there are no conflicts of interest.

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### Data Availability Statement
All data generated or analyzed during this study are included in this published article.

### Ethics approval
This experimental research was planned and approved by the Institutional Animal Care and Use Committee of Shaqra University (ERC-SUF-202400079).

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