International Immunopharmacology 101 (2021) 108274

Contents lists available at ScienceDirect

International Immunopharmacology

journal homepage: www.elsevier.com/locate/intimp

In vitro and *in vivo* therapeutic potentials of 6-gingerol in combination with amphotericin B for treatment of *Leishmania major* infection: Powerful synergistic and multifunctional effects

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ARTICLE INFO

Keywords:
6-Gingerol
Amphotericin B
Leishmania major
Combination therapy
Pathological profiles

ABSTRACT

The ongoing conventional drugs for leishmaniasis treatment are insufficient. The present study aimed to assess 6-gingerol alone and in combination with amphotericin B on *Leishmania major* stages using experimental and *in vivo* murine models. Here, arrays of experimental approaches were designed to monitor and evaluate the 6-gingerol potential therapeutic outcomes. The binding affinity of 6-gingerol and IFN-γ was the basis for docking conformations. 6-Gingerol combined with amphotericin B represented a safe mixture, extremely leishmanicidal, a potent antioxidant, induced a remarkable apoptotic index, significantly increased the expression of the Th1-related cytokines (IL-12p40, IFN-γ, and TNF- α), iNOS, and transcription factors (STAT1, c-Fos, and Elk-1). In contrast, the expression of the Th2-related cytokines was significantly downregulated ($p < 0.001$).

This combination was also potent when the lesion appearance was evaluated following three weeks of treatment. The histopathological and immunohistochemical patterns of the murine model represented clusters of $CD4^+$ and $CD8^+$ T lymphocytes which compressed and deteriorated the macrophages harboring Leishman bodies. The primary mode of action of 6-gingerol and amphotericin B involved broad mechanistic insights providing a coherent basis for further clinical study as a potential drug candidate for CL. In conclusion, 6-gingerol with amphotericin B synergistically exerted anti-leishmanial activity *in vitro* and *in vivo* and potentiated macrophages' leishmanicidal activity, modulated Th1- and Th2-related phenotypes improved the histopathological changes in the BALB/c mice infected with *L. major*. They elevated the leukocyte infiltration into the lesions. Therefore, this combination should be considered for treating volunteer patients with CL in clinical studies.

1. Introduction

Leishmaniasis is a neglected disease produced by parasites of the *Leishmania* genus and spread by the bite of female sandflies. The disease affects 98 tropical and subtropical countries and three territories in over one billion at-risk populations globally. Many clinical presentations are induced by 20 parasitic human *Leishmania* species [1,2], depending on the epidemiological locality, the causative *Leishmania* species, and the

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https://doi.org/10.1016/j.intimp.2021.108274
Received 19 July 2021; Received in revised form 11 October 2021; Accepted 12 October 2021
Available online 21 October 2021

primary reservoir host. While kala-azar or visceral leishmaniasis (VL) is the deadliest type and kills people if not treated, cutaneous leishmaniasis (CL) is the commonly distributed form [1]. Currently, approximately 1.3 million new leishmaniasis cases are reported, three-fourths belong to CL, and the remaining are reported to be VL, leaving up to 30,000 deaths per year and significant public health impacts worldwide [3]. However, the number of leishmaniasis-affected people is more substantial because some cases remain unreported, and misdiagnosis is common [4].

The life cycle of the *Leishmania* species involves a flagellated form and extracellular promastigote stage in the gut of the biological sandfly vector (*Phlebotomus* in the Old World while *Lutzomyia* in the New World). Stationary promastigote forms are engulfed by host macrophages, where they transform into oval and non-motile amastigotes (clinical stage or Leishman bodies). Amastigotes survive and multiply within the phagolysosome compartment despite highly elaborated macrophages defense mechanisms, including hydrolytic enzymes and oxidative stresses. The infection starts following a sandfly takes a blood meal from an infected reservoir host (humans and mammals). During the second blood meal, infected sandflies transmit the infectious metacyclic promastigotes via the skin into the bloodstream of the reservoir host [5]. Clinical manifestations in the form of skin lesions (CL) or involvement of reticuloendothelial system (VL) cause harmful effects, tissue destruction, organ failure, and lethal effect [6].

At present, leishmaniasis has neither effective drugs nor approved efficacious vaccines. Moreover, sandfly vector and reservoir host control strategies are not helpful due to the complexity of the rural and urban life cycle, so many biological vectors, reservoir hosts, and multiple risk factors [7–9]. Therefore, chemotherapy is the primary therapeutic option considered in patients universally [10]. However, conventional synthetic drugs are no longer satisfactory because of parenteral administration, high toxic effects, long-course treatment, parasite resistance, and high cost [11,12]. Hence, such limitation for using conservative synthetic formulations emphasizes an urgent need to develop novel alternative medicinal drugs and their derivatives as active components with varying ranges of the mechanism of action, shorter therapy cycles, high potency, and availability in endemic countries [13].

Plants, natural products, and minerals have been used for a long time to manage various disorders. Plant-based medications and natural products have frequently been used against leishmaniasis [14–17]. As various plant-derived components can have immunomodulatory, antioxidative, antimicrobial, and anti-parasitic impacts, with mild side effects, they may be considered an alternative medication for treating infectious diseases such as leishmaniasis. Experimentally or clinically, the beneficial effects of ginger or its constituents have been indicated in various disorders such as osteoarthritis, rheumatoid arthritis, type 2 diabetes, acute respiratory distress syndrome, ulcerative colitis, airway allergy, neuroinflammation, and experimental autoimmune encephalomyelitis [14]. In addition, the *in vitro* anti-leishmanial effects of the ginger extract have also been indicated [15].

Chemically, ginger (the rhizome of the *Zingiber officinale Family*) has about 400 different ingredients, and the majority of the ginger-related therapeutic effects are attributed to its phenolic compounds, including gingerols, paradols, shogaols, and gingerones [16,17]. In addition, 6-gingerol, as a main pungent ingredient of fresh ginger, exhibits various activities such as anticancer, anti-inflammatory, antioxidant, antimicrobial, antifungal, and neuroprotective effects [16].

Modulation of immune response by herbal medicines and their metabolites has been considered as a promising therapeutic approach. It is now recognized that immunomodulation could provide an alternative to conventional chemotherapy for various disease conditions [18]. Many plant-derived macromolecules include epigallocatechin-3-gallate, curcumin, resveratrol, colchicine, quercetin, capsaicin, genistein, and andrographolide, have displayed strong effects on immune system functions in preclinical assays and highlight their therapeutic potential [18–20].

Ginger has deeply rooted universal, notably in Asian, Arabic, Indian and Chinese cultures used as an old-style medicinal remedy for its spicy and valuable source of therapeutic properties [21]. Ginger possesses a high safety profile and a broad spectrum of biological and pharmacological constituents, such as several bioactive phenolics, non-volatile pungent compounds, acting as antitumor, antimicrobial, antioxidant, anti-inflammatory, antiproliferative activities, and a useful apoptotic index [22]. Other healthful properties such as improving digestion, supporting bone health, relieving pain, and cardiovascular health are also ginger-relevant attributes. In addition, ginger consists of a range of practical modules and lead molecules, including nutrients, vitamins (E, B6, Niacin, and C), and minerals (manganese, iron, selenium, and magnesium), displaying its promising role as a chemopreventive and chemotherapeutic agent [23,24].

6-Gingerol, one of the main components of ginger, is considered a target for new drug development and has the most potent and active constituents. Pharmacological investigations have currently revealed that 6-gingerol has extensive effects on a wide range of cancers. A preclinical meta-analysis of 6-gingerol showed wide ranges of anticancer, anti-inflammatory, antifungal [25,26], and gastroprotective properties [27]. A limited number of *in vivo* studies have proposed that 6-gingerol enables healthy glucose regulation for people with diabetes [28,29]. Several studies have been performed on the effects of 6-gingerol, including leukemia [30], prostate [31], breast [32], skin [33], ovarian [34], lung [35], pancreatic [36], and colorectal [37].

The present study assessed 6-gingerol alone and with amphotericin B on *Leishmania major* stages using experimental and *in vivo* assays. We precisely designed arrays of experimental approaches including molecular docking, anti-leishmanial effects, cytotoxicity, antioxidant and apoptotic levels, gene expression signatures to monitor and evaluate 6-gingerol potential therapeutic outcomes, parasite burden, histopathological and immunohistochemical (IHC) profiles.

2. Material and methods

2.1. Prediction of functional residues of interferon-gamma protein

For predicting the hotspots of functional residues in the structure of interferon-gamma before docking, the "Hotspot" (https://loschmidtchemi.muni.cz/hotspotwizard/) and CASTp (http://sts.bioe.uic.edu/castp/index.html?1ycs) softwares were employed [38,39]. These softwares are able to predict functional residues using the integration of evolutionary, functional, and structural information of several bioinformatics databases.

2.2. Investigation of structural pockets of interferon-gamma protein surface

Delineating and measuring concave surface areas on 3-D structures of proteins are critical before docking. Molgro Virtual Docker software as the cavity search tool (Molegro 2011) was used to identify pockets on protein surfaces and cavities buried inside proteins.

2.3. Protein-ligand docking

The 3-dimensional (3-D) structure of 6-gingerol was attained using PubChem CID 936 [PubChem https://pubchem.ncbi.nlm.nih.gov/compound/Nicotinamide]. The 3-D form of IFN-γ was obtained from the Protein Data Bank (PDB) (ttps://https://doi.org/10.2210/pdb1hig/pdb). Molecular docking studies were performed in Molegro Virtual Docker software in advance before the start of experimentations. Initially, docking was implemented inside a limited search space around possible binding sites. Then, the binding affinity was the reason for selecting docking configurations using Molegro Molecular Viewer 2.5.0 (Molegro ApS, Aarhus, Denmark) for graphical illustration.

2.4. Chemical preparation

To prepare a stock solution of each drug, 2000 μg of 6-gingerol (Sigma-Aldrich Co., Germany) and amphotericin B (Health Biotech ltd. India), this brand is used for intravenous infusion in humans, this product is completely soluble in water [40,41]) were dissolved in 1 mL sterile distilled water to prepare suitable concentrations (10-fold concentrated drug 125, 250, 500, 1000 and 2000 μg /mL) to achieve final concentrations of 12.5, 25, 50, 100, and 200 μg/mL and also for different combinations to achieve final concentrations of 25 + 25, 50 + 50, and 100 + 100 and 200 + 200 μg/mL (only for intra-macrophage amastigotes) as prepared just before experimentations to assess the combinatory effect of 6-gingerol and amphotericin B. The doses of drugs were selected according to the dose–response assays. No attempt was made to prepare higher concentrations due to maximum strength at the latter combinatory concentration.

Roswell Park Memorial Institute medium (RPMI1640), MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide), a tetrazole (MTT), fetal calf serum (FCS), penicillin/streptomycin, butylated hydroxyanisole (BHA), and zinc sulfate, 2,2-Diphenyl-1-picrylhydrazyl (DPPH) were procured from Sigma-Aldrich, France.

2.5. Parasite culture

Initially, *L. major* strain (MHOM/IR/Mash2) was seeded in Novy-MacNeal-Nicolle (NNN) culture medium and then in RPMI1640, which was added with 100 IU/mL of penicillin, 100 μg/mL of streptomycin (Sigma, Germany) and 10 % v/v heat-inactivated fetal bovine serum (FBS) at 24 ± 1 °C then incubated for half an hour. The strain was retained at −20 °C for advanced experimental works. The promastigote forms were set at 25 °C. The cumulative proportion of promastigotes was measured every 24 h, and the number of the promastigotes was counted in a 10 μL using a Neubauer slide compartment.

2.6. Macrophage culture

A murine macrophage cell line (J774-A1) was obtained from Pasteur Institute (Tehran, Iran) and cultured in Gibco Dulbecco's Modified Eagle's Medium (DMEM), with 10 % FBS, 1 % penicillin and streptomycin, at 37 °C, and 5 % CO2. The promastigotes were detected by an inverted microscope every 24 h, the medium containing the organism was transferred to the culture flask.

2.7. Assessment of the anti-promastigote activity

The MTT test was used to evaluate the anti-promastigote effect of 6-gingerol, amphotericin B, or their combination against the promastigote form of *L. major*. Initially, 90 μL of the promastigotes (10^5 cells/mL) from the exponential growth stage was added to a 96-well microplate. Then, 10 μL of different concentrations of 6-gingerol and amphotericin B (0–200 μg/mL), or their combination, was transferred to each well and then incubated at 25 °C for 72 h. Subsequently, 10 μL of the MTT with 10 mg/mL concentration was assigned to each well and immediately incubated at 24 ± 1 °C for 3 h. The organisms were also cultured without any drug as negative control and a complete medium without drugs and promastigotes as blank. Tests were carried out in three sets. Lastly, the isopropanol alcohol was used to halt the reaction, and the OD absorbance was measured for each well at 490 nm by an enzyme-linked immunosorbent assay (ELISA) reader (Bio Tek-ELX800). The IC_{50} value was calculated in SPSS software version 20 by probit test.

2.8. Assessment of the anti-amastigote activity

One cm^2 coverslip was placed in each well of 8-compartment slides (Lab-Tek, Nalge Nunc International NY, USA). Then, 200 μL of macrophage suspension (10^6) was incubated at 37 °C in 5 % CO2 for 24 h. Additionally, 200 μL (10^7) promastigotes were transferred to the macrophages in the metacyclic growth phase. Thus, *Leishmania*: macrophage ratio was 10:1, respectively, and was again incubated in the same condition for 24 h. Further, 40 μL of varying concentrations of 6-gingerol, amphotericin B, or their combination were added to the infected macrophages at the same conditions for 72 h.

Afterward, the slides were fixed with methanol alcohol, stained with standard Giemsa, and visualized by an optical microscope. Drug action was assessed by counting amastigotes (Leishman bodies) in the cells by examining 100 cells. Every test was done thrice.

2.9. Assessment of the cytotoxic effects of 6-gingerol and amphotericin B

The murine cell line (5×10^5) was cultured with different concentrations of the investigated drugs (0–200 μg/mL) in 96-well microplates (at 37 °C in 5 % CO2 for 72 h) to evaluate the cytotoxicity of 6-gingerol, amphotericin B, or their combination using MTT assay. The wells which contained medium and parasites without drugs were considered as a negative control group. After 72 h of exposure, 10 μL of the MTT with 10 mg/mL concentration was transferred to each well. Then, the microplates were incubated for three h for the emergence of formazan crystals. Afterward, 100 μL of isopropanol alcohol was added in order to solve formazan crystals. The exposure of isopropanol alcohol was retained for one h, and then ELISA-reader was used to read optical density at 490 nm. Finally, the colorimetric MTT test measured cell viability. The data were shown as the percentage of dead cells in cultures treated with 6-gingerol, amphotericin B, or both drugs and compared with non-treated macrophages.

2.10. Assessment of the antioxidant activity of 6-gingerol

The scavenging effect of free radical 2, 2-diphenyl-1- picrylhydrazyl (DPPH) was carried out consistent with the method described elsewhere [42]. The present study was based on the reduction of DPPH as a constant free radical. DPPH free radicals display an extreme value of optical density at 517 nm. The degree to which the absorbance drops is directly linked to the degree of reduction of DPPH. The following formula calculated free radical scavenging: = (absorbance of control- absorbance of the sample) / (absorbance of control) × 100.

2.11. Flow cytometry analysis

The flow cytometry examination employing PE Annexin V Apoptosis Detection Kit I (BD Pharningen ^{TM}) was performed to specify apoptosis and cellular viability ranges of *L. major* promastigotes exposed to drugs (6-gingerol, amphotericin B, or a combination). Briefly, 1×10^6 promastigotes were seeded in a 2-mL microtube in the existence of 100 μL of several concentrations of different drug treatments. Mixtures were incubated at 25 °C for 72 h, and promastigotes were washed by PBS (pH 7.2) and re-suspended in 1 mL binding buffer. Then 100 μL of the above solution was added to a 5-mL glass tube, and 5 μL of Annexin V and 5 μL 7-AAD stain were added to each tube and kept in darkness at 25 °C for 20 min. Accordingly, the apoptosis rate in the treated parasites with a single drug or both drugs was analyzed using a flow cytometer. In addition, the apoptosis rate was equated between different treatment groups (0–200 μg/mL).

2.12. Quantitative real-time PCR

The quantified expressions of IFN-γ, IL-12p40, TNF-α, iNOS, STAT1, c-Fos, Jak1, Elk1, IL-4, TGF- β, and IL-10 were identified utilizing real-time PCR (qPCR) test on murine macrophage cell line (J774-A1). Briefly, the total RNA was extracted from different treated and negative control groups with the RNeasy mini kit (Qiagen, Chatsworth, CA, USA) according to the producer's protocol. The RNA concentration (ng) and purity were determined according to the OD's measurement at 260 nm

and 280 nm by a spectrophotometer system (NanoDrop ND-1000, Thermo Scientific, Wilmington, DE, USA). The extracted RNA was converted to cDNA using Takara Prime Script™ RT reagent kits (Takara Bio, Inc., Japan). The reverse transcription process was completed on 500 ng of the total RNA at 37 °C for 20 min. The qPCR reaction was achieved in duplicate with the Rotor-Gene Cycler system (Rotor-Gene 3000 cycler, Corbett, Sydney, Australia) using an SYBR Green master mix (SYBR Premix Ex Taq™ II, Takara Bio, Inc., Shiga, Japan).

Table 1 shows the template and control gene sequences. Initially, a holding treatment was done at 95 °C for 1 min, and then the cDNA was augmented by 40 three-step rounds (10 *sec* at 95 °C for denaturation of DNA, 15 *sec* at 58 °C for primer annealing, and 20 *sec* at 72 °C for extension). The last temperature of elongation was 65 °C for 1 min. Expression rates were measured compared to the expression profile of the selected reference gene. The ΔCT was measured employing the following equation: [ΔCT = CT (target) - CT (reference)]. Gene expression profile was specified by the $2^{-\Delta Ct}$ method. Moreover, the fold change was measured through the comparative threshold technique ($2^{-\Delta\Delta CT}$).

2.13. Infection of BALB/c mice, treatment programs, and determination of the parasite burden and cure rate

L. major standard strain was maintained in BALB/c mice. The hind footpad of each mouse was inoculated with 2×10^6 metacyclic promastigotes in 50 μL sterile PBS. The mice were randomly allocated into 4 groups of 5 mice each, treated with 100 μL 6-gingerol, 100 μL amphotericin B alone, 6-gingerol plus amphotericin B or an untreated control group which received the same volume of the vehicle. The treatment schedule was continued for 21 days. Insulin syringes (26 G needles) were used for intraperitoneal (IP) injection. All tests on mice were carried out in agreement with the recommendation for Research on Laboratory Animals, permitted by the Ethics Committee of the Ministry of Health and Medical Education of Iran. In addition, this investigation was obtained approval from the Ethics Committee of Kerman University of Medical Sciences (Contract no. 400,000,245 and Ethics Code: IR.KMU.REC.1400.163).

Table 1
The specific primers and reference gene (GAPDH and HPRT) sequences.

Template	Forward and reverse sequences (5′-3′)		Product size (bp)
IL-4	Forward	5-GGTCTCAACCCCCAGCTAGT-3	101
	Reverse	5-GCCGATGATCTCTCTCAAGTGAT-3	
IFN-γ	Forward	5-ACAGCAAGGCGAAAAAGGATG-3	106
	Reverse	5-TGGTGGACCACTCGGATGA-3	
TGF-β	Forward	5-CCACCTGCAAGACCATCGAC-3	112
	Reverse	5-CTGGCGAGCCTTAGTTTGGAC-3	
iNOS	Forward	5-ACATCGACCCGTCCACAGTAT-3	89
	Reverse	5-CAGAGGGGTAGGCTTGTCTC-3	
GAPDH	Forward	5-AGGTCGGTGTGAACGGATTTG-3	95
	Reverse	5-GGGGTCGTTGATGGCAACA-3	
HPRT	Forward	5-TCAGTCAACGGGGGACATAAA −3	142
	Reverse	5-GGGGCTGTACTGCTTAACCAG −3	
Stat1	Forward	5-GCTGCCTATGATGTCTCGTTT-3	154
	Reverse	5-TGCTTTTCCGTATGTTGTGCT-3	
Jak1	Forward	5-ACGCTCCGAACCGAATCATC-3	123
	Reverse	5-GTGCCAGTTGGTAAAGTAGAACC-3	
c-Fos	Forward	5-CGGGTTTCAACGCCGACTA-3	94
	Reverse	5-TGGCACTAGAGACGGACAGAT-3	
Elk1	Forward	5-TTGTGTCCTACCCAGAGGTTG-3	168
	Reverse	5-GCTATGGCCGAGGTTACAGA-3	
IL-12p40	Forward	5-TGGTTTGCCATCGTTTTGCTG-3	171
	Reverse	5-ACAGGTGAGGTTCACTGTTTCT-3	
TNF-α	Forward	5-CAGGCGGTGCCTATGTCTC-3	161
	Reverse	5-CGATCACCCCGAAGTTCAGTAG-3	
IL-10	Forward	5-CTTACTGACTGGCATGAGGATCA-3	134
	Reverse	5-GCAGCTCTAGGAGCATGTGC-3	

2.14. Lesion size assessment

After three weeks of therapy, the lesion size resolution was achieved by examining the diameter of the mice footpad's induration by a digital Vernier Caliper. In addition, the average lesion size was recorded by measuring the left hind footpad length and width in the different treatment BALB/c mice groups relative to that of the non-infected contra-lateral right hind footpad in all groups [43].

2.15. Histopathological examination

BALB/c mice popliteal lymph nodes were fixed in 10 % formalin and embedded in paraffin for histopathological analysis. Five μm tissue sections were prepared, stained with hematoxylin and eosin (H and E). Microscopic examinations calculated the number of parasite burdens based on the Ridley scoring system [44]. A Leica Orthoplan microscope took all images.

2.16. Immunohistochemical (IHC) evaluation to identify the patterns of infiltrated leukocytes

The IHC profile was designed to explore the nature of different inflammatory cell-induced against the *Leishmania* parasite in the studied tissue. First, tissue sections of 5 μm width were prepared and salinized to enhance the adherence of cells. Then, sections were gradually chilled to ambient temperature. The succeeding slides were washed quickly in tris buffered saline at pH 7.4 and stained with four monoclonal antibodies against cell surface antigens of leukocytes [45], including anti-CD4 (code MU421-UC; clone 4B12), anti-CD8 (code MU422-UC), anti-CD20 (code MU238A-UC; clone L26), and anti-CD68 (code MU416-UC; clone KP1) (BioGenex Company) [45], The 3, 3′-diaminobenzidine (DAB) chromogen was used for grading the expression of the markers. An optical microscope and a hematology cell analyzer considered various cell phenotypes (cells/mm2). All essential microscopic fields were counted in each section at a magnification of 400X [46].

2.17. Statistical analyses

The anti-leishmanial effects of drugs were evaluated by the mean number of amastigotes per macrophage of 100 macrophages as triplicates. The data were analyzed by SPSS statistical package version 20.0 (SPSS Inc., Chicago, IL, USA). We used the *t*-test and ANOVA to study the differences between the treated and negative control groups. Moreover, $p < 0.05$ was set as statistically significant. The IC_{50} values (50 % inhibitory concentrations) were intended in SPSS by probit test. The *t*-test was applied to define the changes between IC_{50} in two stages of *L. major* life cycle. The selectivity index (SI) was calculated according to the equation: CC_{50} for peritoneal macrophage cells/$IC_{50} \geq 1$, non-toxic [47].

3. Results

3.1. Docking analyses indicated the capability of 6-gingerol for binding to IFN-γ

In assessing the results of available amino acids by Hotspot software, amino acids Lys14, Met135, and Leu136 were predicted. Based on the algorithm of this software, these amino acids were highly mutable residues in catalytic pockets and access tunnels.

3.2. Structural pockets prediction of protein surface

The structural basis for functional studies is determined by specific topological and geometric features, including surface pockets. Identifying these features can be fruitful in the treatment development process. The result shows that IFN-γ consists of a central pocket and 4

cavities (Fig. 1A). The predicted amino acids in pocket formation were as follows: *residues (*Tyr5, Val6, Ala9, Glu10, Leu12, Lys13, Phe16, Ala18, Val23, Ala24, Asn26, Gly27, Thr28, Leu29, Phe30, Leu31, Leu34, Arg43, Gln47, Ile50, Val51, Phe53, Tyr54, Leu57, Phe58, Phe61, Ile74, Asp77, Met78, Lys81, Phe82, Lys131, Arg132, Ser133, Gln134, Met135, Leu136, and Phe137*)*.

After docking, the 2-D interaction diagrams were evaluated. These diagrams describe highly hydrophobic cavities consisting of a number of adjacent hydrophobic residues and ligand-cavity hydrogen bonds. In fact, hydrogen bonds and hydrophobic interactions are the main contributing forces in the binding energy of the ligand and target protein. In the present study, the amino acids involved in the interplay between the central pocket of IFN-γ and 6-gingerol metabolites were determined by the online server Ligand Interaction Profiler (PLIP). The interaction between 6-gingerol and IFN-γ is mainly mediated by hydrogen bonds with Ser133 and Gln134 residues of the central pocket. Regarding steric interactions, 6-gingerol interact with Ser133, Gln134, and Lys13 amino acids of IFN-γ, respectively (Fig. 1B).

According to the molecular docking results, 6-gingerol binds to IFN-γ (Fig. 1C) with the active site residues (Ala112, Ala116, Arg76, Gln 69, Ile116, Lys109, Phe102, Phe103, Phe113, Ser68, and Ser72) (Table 2 and Fig. 1D). In addition, it demonstrated a MolDock score of 2.21159 kcal/mol (Table 3).

3.3. 6-Gingerol, amphotericin B, and their combination exerted anti-leishmanial effect against the promastigote form of L. major

The total mean viability values of *L. major* promastigotes treated by various concentrations of 6-gingerol, amphotericin B, or their combination are presented in Fig. 2. The results of 6-gingerol, amphotericin B alone, or combination showed a notable anti-leishmanial effect against the promastigote form of *L. major* in a dose-dependent response ($p < 0.001$).

3.4. 6-Gingerol, amphotericin B alone, and their combination exerted antileishmanial effect against the amastigote form of L. major

The activity of the above drugs was estimated by the average number of amastigotes in the macrophages. Different concentrations of 6-gingerol, amphotericin B alone (Table 4), and their combination (Table 5) significantly reduced the number of amastigotes in infected macrophages compared to untreated macrophages ($p < 0.001$).

Overall, the IC_{50} values for promastigotes were significantly higher than those for the clinical-stage amastigotes ($p < 0.001$). 6-Gingerol combined with amphotericin B exhibited the least IC_{50} value (12.96 μg/mL) compared to 6-gingerol alone (35.76 μg/mL). There was no cytotoxicity associated with drugs used against intra-macrophage amastigotes (Table 6).

3.5. 6-Gingerol and amphotericin B alone or their combination did not exert a cytotoxic effect on macrophages

Different concentrations of 6-gingerol, amphotericin B (0, 12.5, 25, 50,100, and 200 μg/mL) were assessed for their effect on the macrophage cell line. The selectivity index (SI) values for 6-gingerol, amphotericin B, and their combination were 20, 8.2, and 21, respectively (Table 6). None of the drugs exhibited a significant cytotoxicity effect against the mammalian macrophages.

3.6. 6-Gingerol exhibited potent antioxidant activity

The radical scavenging action of 6-gingerol and BHA on DPPH was estimated by measuring the compounds' hydrogen donations (Fig. 3). The activity followed a dose–response outcome. The overall IC_{50} value of 6-gingerol and BHA were 79.09 μg /mL and 104.12 μg/mL, respectively. There was a significant difference ($p < 0.05$) between the 6-gingerol antioxidant action and that of BHA ($p < 0.05$).

3.7. 6-Gingerol, amphotericin B alone and their combination induced apoptosis in L. major

Treatment of promastigotes with 6-gingerol, amphotericin B alone, and their combination led to apoptosis in *L. major* (Fig. 4). All concentrations of the three treated sets presented significant differences relative to the negative control group ($p < 0.001$). Amphotericin B significantly increased the apoptotic level compared to 6-gingerol, except at 12.5 μg/mL concentration. Also, all 6-gingerol plus amphotericin B combined concentrations showed significantly higher apoptosis

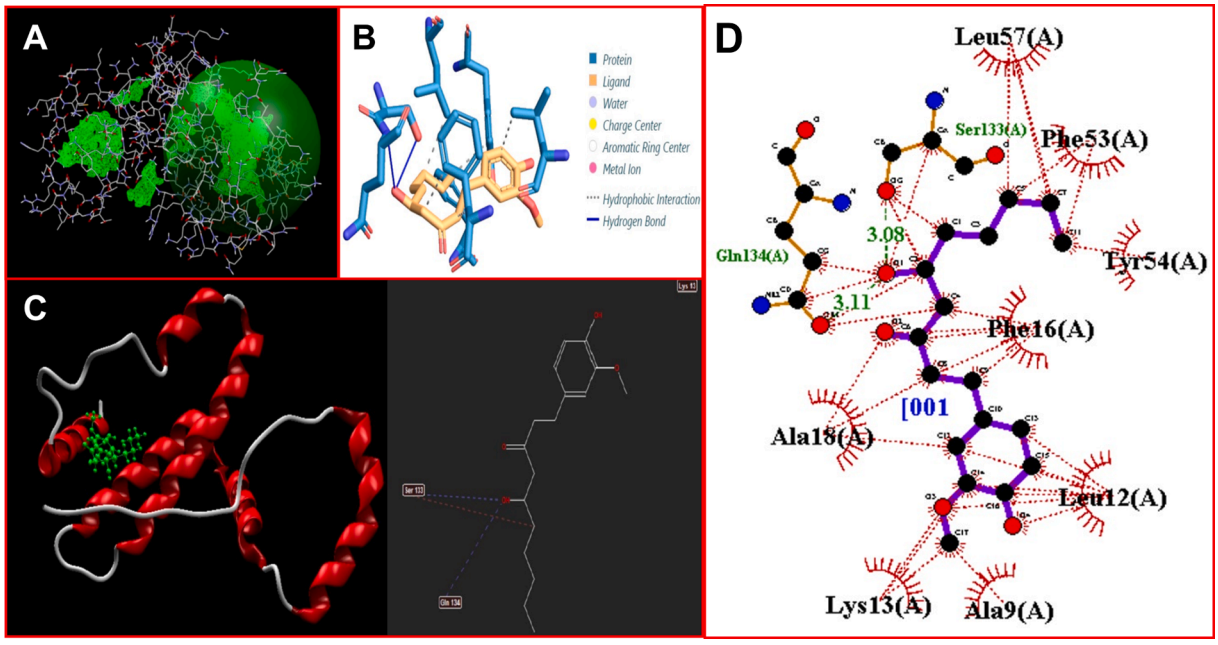

Fig. 1. Docking. A) IFN-γ consists of a central pocket and 4 cavities. B) Predicted amino acids in pocket formation by PLIP web tool. C) Molecular docking by Molgro Virtual Docker software. D) 6-Gingerol binds to IFN-γ with the active site residues by LIGPLOT program.

Table 2
Contribution of the IFN-γ residues/molecules.

Hydrophobic Interactions									
Index	Residue	AA	Distance	Ligand Atom	Protein Atom				
1	12A	LEU	3.64	2279	163				
2	16A	PHE	3.35	2268	248				
3	16A	PHE	3.25	2273	246				
4	16A	PHE	3.74	2272	245				
5	30A	PHE	3.91	2275	433				
6	54A	TYR	3.38	2275	845				
7	57A	LEU	3.50	2269	906				
Hydrogen Bonds									
Index	Residue	AA	Distance H-A	Distance D-A	Donor Angel	Protein donor	Side chain	Donor Atom	Acceptor Atom
1	133A	SER	2.15	3.08	157.19	×	✔	2261[O3]	2180[O3]
2	134A	GLN	3.44	3.89	108.44	✔	×	2186[Nam]	2261[O3]

Table 3
Molecular docking score.

Type	Heavy atoms	Total	ELntra	EPair
All atoms	21	2.21159	2.21159	0

levels than amphotericin B alone concentrations as the positive control drug ($p < 0.0001$).

3.8. 6-Gingerol, amphotericin B alone, and their combination promoted the gene expression of the T cell-related parameters

The gene expression of T cell-mediated immune responses was analyzed in 6-gingerol, amphotericin B alone, and 6-gingerol plus amphotericin B treated macrophages and untreated control cells. The expression of Th1 cell-related parameters (including IFN-γ, IL-12p40, TNF-α, and iNOS), Th1-related transcription factors (including STAT1, c-Fos, and Elk-1), and Th2 cell-related parameters (including IL-4, IL-10, and TGF-β) was designated in Figs. 5-7, respectively. The expression of Th1-related parameters and transcription factors was significantly elevated in the 6-gingerol or amphotericin B-treated groups compared with untreated cells.

The expression levels in Th1 and Th2 cytokines and transcription factors in the 6-gingerol group were the same as the amphotericin B group. However, the combination group (6-gingerol plus amphotericin B) represented a significant increase of Th1 cytokines expression (Fig. 5) and transcription factors (Fig. 6). In contrast, a significant decrease in Th2 cytokines expression was observed (Fig. 7) compared to the amphotericin B group at the same concentrations ($p < 0.001$). Fig. 8 displays the impacts of the up-regulation of the Th1-related cytokines and transcription factors and down-regulation of the Th2-related cytokines on the parasite survival.

3.9. Correlation of the gene expression of Th1- and Th2-related parameters in the 6-gingerol and/or amphotericin B-treated macrophages

As presented in Fig. 9, a significant positive correlation was detected

Table 4
Comparison of the effects of different concentrations of 6-gingerol and amphotericin B alone on the mean number of intra-macrophage amastigotes.

Concentration (μg/mL)	6-Gingerol		Amphotericin B	
	Mean ± SD	P-value	Mean ± SD	P-value
0.0 (Control)	39 ± 4.2	NR*	39 ± 4.2	NR*
12.5	27 ± 1.41	$P < 0.01$	32.5 ± 0.7	$P < 0.05$
25	21.5 ± 2.1	$P < 0.001$	29 ± 0.0	$P < 0.01$
50	17.5 ± 0.7	$P < 0.001$	22.5 ± 2.1	$P < 0.001$
100	12.5 ± 2.1	$P < 0.001$	11 ± 1.41	$P < 0.001$
200	6.2 ± 0.8	$P < 0.001$	4.5 ± 1.1	$P < 0.001$

*Not related.

Table 5
Comparison of the effects of different concentrations of 6-gingerol and amphotericin B combinations on the mean number of intra-macrophage amastigotes.

Concentration (μg/mL)	6-Gingerol + amphotericin B	
	Mean ± SD	P-value
0 (Untreated control)	28.56 ± 3.8	NR*
25 + 25	12.46 ± 0.15	$P < 0.001$
50 + 50	9.67 ± 0.6	$P < 0.001$
100 + 100	0	$P < 0.001$

*Not related.

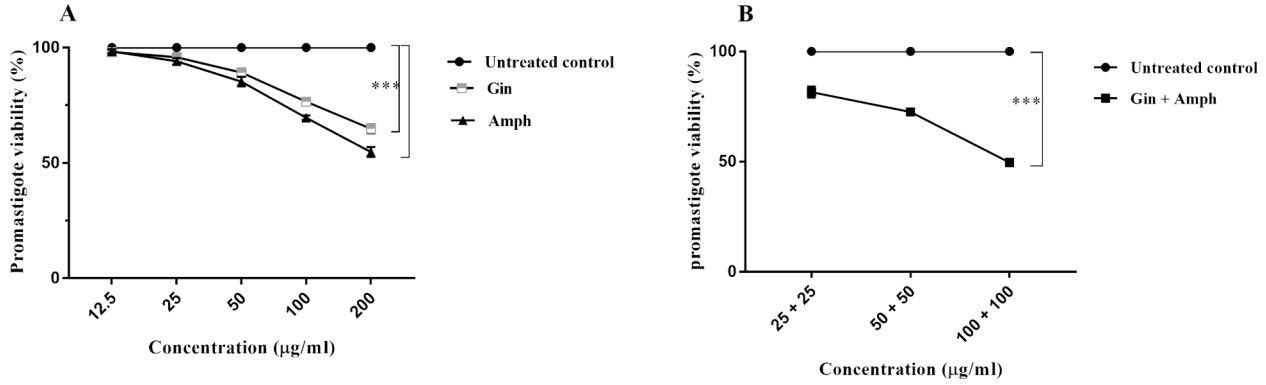

Fig. 2. Comparison of the overall mean viability values of *L. major* promastigotes (non-clinical stage) treated with different concentrations of 6-gingerol (Gin), amphotericin B (Amph), or their combination (Gin + Amph) by colorimetric assay (***$p < 0.001$).

Table 6
Evaluating the IC_{50} values of 6-gingerol, amphotericin B alone, and 6-gingerol plus amphotericin B combination against amastigote and promastigote forms of *L. major* compared with amphotericin B as positive control drug and the CC_{50} values of the drugs on macrophage using the SI index.

Drugs	Amastigote		Promastigote		Macrophage	[c]SI
	[a]IC_{50} ± SD (μg/mL)	P-value	[a]IC_{50} ± SD (μg/mL)	P-value	[b]CC_{50} (μg/mL)	(Selectivity Index)
Amphotericin B	54.21 ± 5.3	NR	260.2 ± 186	NR	446.86	8.2
6-Gingerol	35.76 ± 6.6	$P < 0.001$	810.2 ± 207	$P < 0.001$	715.45	20
6-Gingerol & amphotericin B	12.96 ± 0.02	$P < 0.001$	174.62 ± 22	$P < 0.01$	272.65	21

NR: Not related
[a] IC_{50} = Drug concentration that inhibited 50 % of promastigotes and amastigotes growth.
[b] CC_{50} = Cytotoxic concentration that inhibited 50 % of macrophages growth.
[c] SI = Selectivity index (CC_{50} of macrophage/ IC_{50} of amastigote).

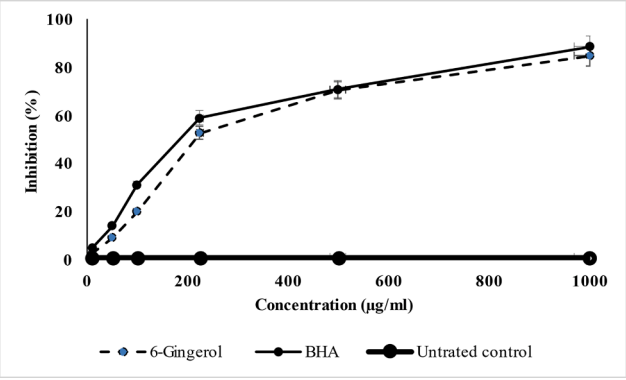

Fig. 3. Scavenging effects of 6-gingerol on 1, 1-diphenyl-2-picrylhydrazyl (DPPH) free radicals compared to butylated hydroxyanisole (BHA) as a standard control. Data expressed as means ± SD of triplicate experiments.

within cytokines of the same pathway (Fig. 9A, B, C, and D) ($p < 0.001$);. However, a significant negative correlation was identified between IL and 4 and IFN-γ (Fig. 9E) ($p = 0.008$), as well as TNF-α and TGF-β (Fig. 9F) ($p = 0.006$) at different concentrations.

3.10. Treatment with 6-gingerol, amphotericin B alone, and their combination promoted recovery in the BALB/c mice infected with L. major

The gross appearance of lesions following treatment in three mice groups (6-gingerol, amphotericin B, and untreated control) showed active lesions, unlike mice that received a combination of 6-gingerol and amphotericin B that represented complete cure (Fig. 10).

3.11. Treatment with 6-gingerol, amphotericin B alone, and their combination improved the histopathological changes in the L. major-infected BALB/c mice

In the negative control group, the structure of the lymph nodes was disorganized. In the cortex regions, the follicles were destructed, and some cells showed necrotic changes with pyknotic, karyorrhexis, and dark elongated nuclei. In the medulla areas, sinus histiocytosis was observed. Other inflammatory cells such as lymphocytes and plasma cells were present. Sections on H&E staining showed complete effacement of nodular architecture by diffuse proliferation of the mononuclear cells. They were composed of primary lymphocytes and few histiocytic cell aggregations, while no intracytoplasmic Leishman bodies were seen. The histiocytes had large pale cytoplasm containing numerous Leishman bodies. Also, the medulla was edematous (Fig. 11A). In the amphotericin B group, the dominant lesion was moderate sinus histiocytosis with low parasitic loads (Fig. 11B).

In contrast, in the 6-gingerol group, the structure of the lymph node cortex was preserved in the medulla, some inflammatory cells such as histiocytes, lymphocytes, and plasma cells were infiltrated. Histiocytes had a scant number of amastigotes in their cytoplasm (Fig. 11C). In combination with the 6-gingerol and amphotericin B group, the lymph nodes showed minimal histopathological changes. Sinus histiocytosis was scarce, and the parasitic load was rare (Fig. 11 D and Table 7).

3.12. Treatment with 6-gingerol and amphotericin B combination modulated the infiltration of the leukocytes into the lesions of the L. major-infected BALB/c mice

The immunohistochemical profile was used as complementary data to approve the results. Fig. 12 showed an increased number of $CD4^+$ T, $CD8^+$ T lymphocytes, $CD20^+$ B cells, and decreased $CD68^+$ cells (M2 macrophages) in mice received a combination of 6-gingerol plus amphotericin B.

4. Discussion

There has been an increasing demand for evaluating and applying herbal drugs as preventive and therapeutic options. A recent study indicates that nearly 80 % of the world's residents trust traditional plant medicines for their health care desires [48]. However, measures to control leishmaniasis have not been efficient, and attempts to develop effective vaccines are still far from successful. Therefore, improved and rational measures for drug development are still needed. Recent progress in molecular biology concerning genomic modulation and molecular signaling pathways has greatly facilitated drug design, drug delivery, and immunotherapy to provide newer intervention strategies against parasites.

The ongoing conventional regimen for leishmaniasis suffers from adverse effects, high costs, severe pain, and parasite resistance [13,49]. Therefore, more attempts need to provide novel compounds that are affordable, more effective, less toxic targets, and with different action mechanisms. 6-Gingerol, as an efficient ginger-derived compound, has been used against many infectious and non-infectious complications [23,24]. 6-Gingerol showed powerful inhibitory effects against *L. major in vitro* and *in vivo* murine models in this study. The SI as a marker of toxicity demonstrated that 6-gingerol is highly safe for mammalian macrophages [47] as the central harboring cell of the *Leishmania* parasites. Similarly, amphotericin B as an approved drug has long been used against various disease conditions, including leishmaniasis, and proved relatively safe *in vitro* assays (SI = 8.2).

The results demonstrated that the effect of 6-gingerol was more profound on the clinical stage (amastigotes) than on promastigotes. Although intracellular, amastigotes have shown to be more susceptible to conventional drugs relative to promastigotes [20,50]. This stage is readily able to reduce pentavalent antimonial agents to trivalent form, unlike promastigotes. Amastigotes can simply concentrate therapeutic drugs as these two stages are different biologically and molecularly [51]. More susceptibility of the intra-macrophage stage to oxidative stress exerted by the drug in use is the basis of parasitic chemotherapy. Although 6-gingerol alone was effective against both promastigotes and amastigotes alone, it displayed more destructive action when combined

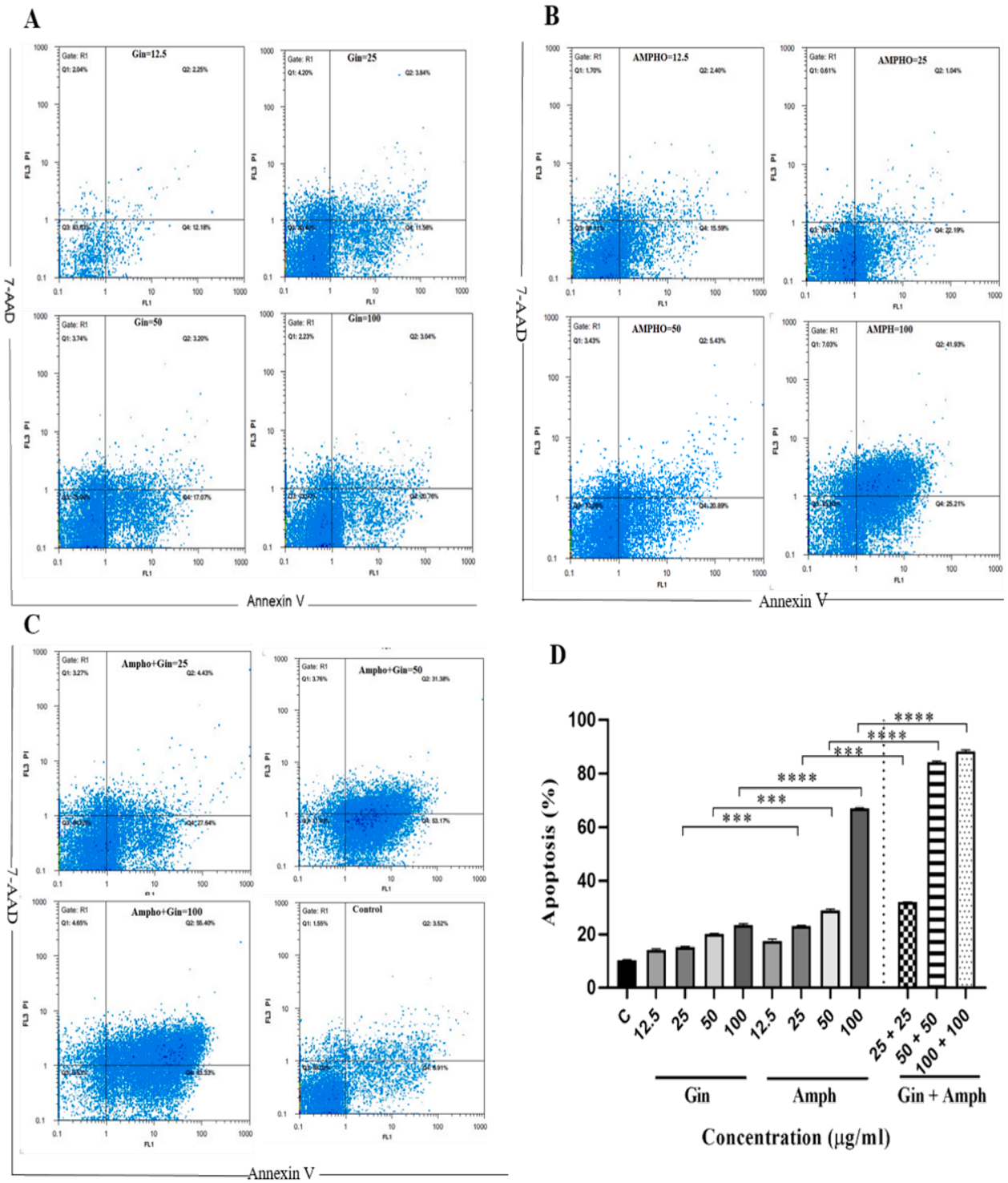

Fig. 4. The apoptotic profiles of the *L. major* promastigote with annexin V at different concentrations of gingerol (A), amphotericin B (B), and Amph plus Gin combination (C). The combination group represented the highest apoptotic level compared to the others (D). Error bars are SD (***$p < 0.001$ and ****$p < 0.0001$).

with amphotericin B in experimental and *in vivo* assays.

The immunological control of *L. major*-related CL mainly depends on the Th1 cell-mediated immune responses, leading to macrophage stimulation and killing intra-macrophage amastigotes (5). The inhibitory effect obtained in this study is superior to those found by other phenolic compounds on *Leishmania* species [52–54]. Moreover, we designed a vast broad of gene expression signatures related to Th1- and Th2 cell responses to elucidate the action mode of the 6-gingerol and amphotericin B in potentiating anti-parasite immune responses. We hypothesized that an evaluation of genes variably expressed between different treatment groups might detect genes involved with treatment outcome and could be helpful as targets for therapeutic screening in providing a rational basis for drug selection in CL.

Although the host immune responses' elimination of the *Leishmania* parasite is very complicated and multifactorial, the intrinsic susceptibility or resistance has been closely associated with distinct subtypes of $CD4^+$ T-helper cells [52,55]. It is well-known that cytokines have an essential part in the pathogenesis and host resistance of leishmaniasis.

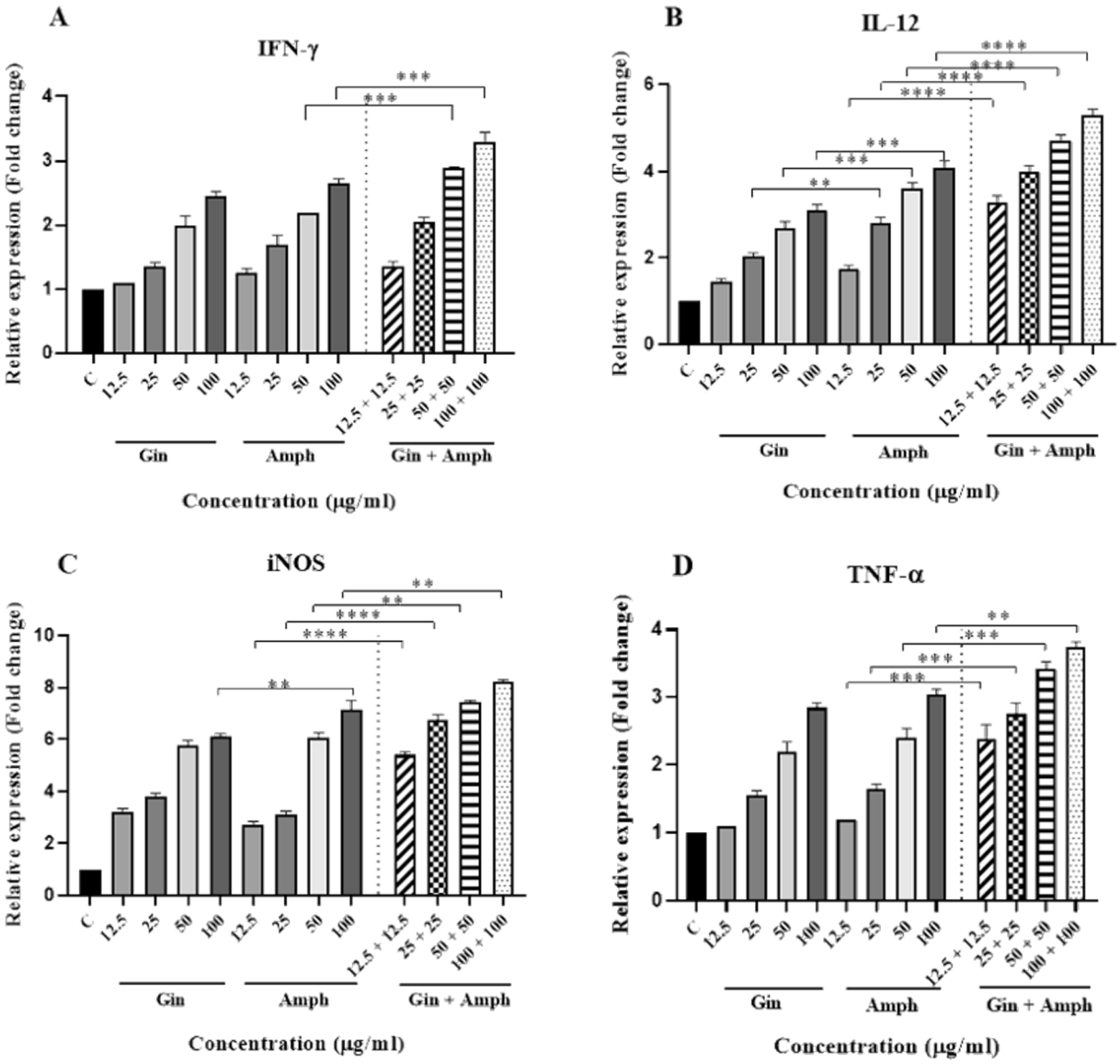

Fig. 5. The gene expression of the Th1-related parameters in macrophages treated with different concentrations of Gin (6-gingerol), amphotericin B (Amph), and Gin + Amph compared to the untreated group. IFN-γ (A), IL-12 (B), iNOS (C), and TNF-α (D). Error bars are SD (**$p < 0.01$, ***$p < 0.001$ and ****$p < 0.0001$). Each test was conducted in triplicate.

Each of these Th cell subsets is characterized by a particular pattern of gene expression, including signature cytokines, regulatory factors, and cell surface receptors. 6-Gingerol triggered the gene expression of the signaling pathways that enable the host cells to combat the *Leishmania* multiplication potentials, resulting in the killing of the organism. It is well-known that resistance to the leishmanial agent is firmly related to Th1 proliferation and generation of pro-inflammatory cytokines such as IL-12p40, IFN-γ, and TNF-α, leading to macrophage activation and parasite's death. Th1 response has a crucial role in immunoprotection against CL [55]. In addition, the current docking outcome demonstrated that 6-gingerol could bind to IFN-γ to form a stable complex, with a score of −92.7086 kcal/mol, which might move the immune response in the direction of the Th1 phenotype [56].

TNF-α is often induced by phagocytic cells that play a vital role in leishmanial clearance by introducing NO synthesis in macrophages [55]. This cytokine is also able to enhance Th1/IFN-γ responses against *L. major* infection. It is worth mentioning that TNF-α and IFN-γ employ their killing activity via macrophage induction to stimulate NO production by iNOS [55,57,58]. 6-Gingerol promoted the gene expression of iNOS and Th1-related cytokines in infected macrophages with *L. major* amastigotes. Consistent with Th1, STAT1 is stimulated in reply to IFN-γ-induced signaling and strengthens the Th1 subtype pathway in a helpful response loop [59]. The *in vitro* biological effect of crude extract of ginger has already been studied. The water extract was able to stimulate macrophages via NO production against *L. amazonensis* [15].

IL-12p40 signaling triggers STAT4 to adjust different features of the Th1 genetic package positively. Thus, STAT1 and STAT4 have clarified their characters in adaptable Th1 signature genes [60]. NK cells could also be activated by cytokines such as IFN-α/β, IL-12p40, IL-18, TNF-α, and IL-1β, alone or in a synergistic mixture by binding to different receptors and stimulating signaling pathways like JAK/STAT in the circumstance of IFN-γ [61].

Amphotericin B combined with paromomycin or miltefosine could synergistically inhibit the intracellular amastigotes, decrease the number of extracellular promastigotes and limit the disease load over a long period [62]. Another study observed a combinatory effect between imiquimod and amphotericin B because endogenous IFN-γ is involved with the leishmanicidal response to amphotericin B [63]. Moreover, imiquimod serves similarly to IFN-γ to induce the release of NO from macrophages. The combinatory mixture could also progress the

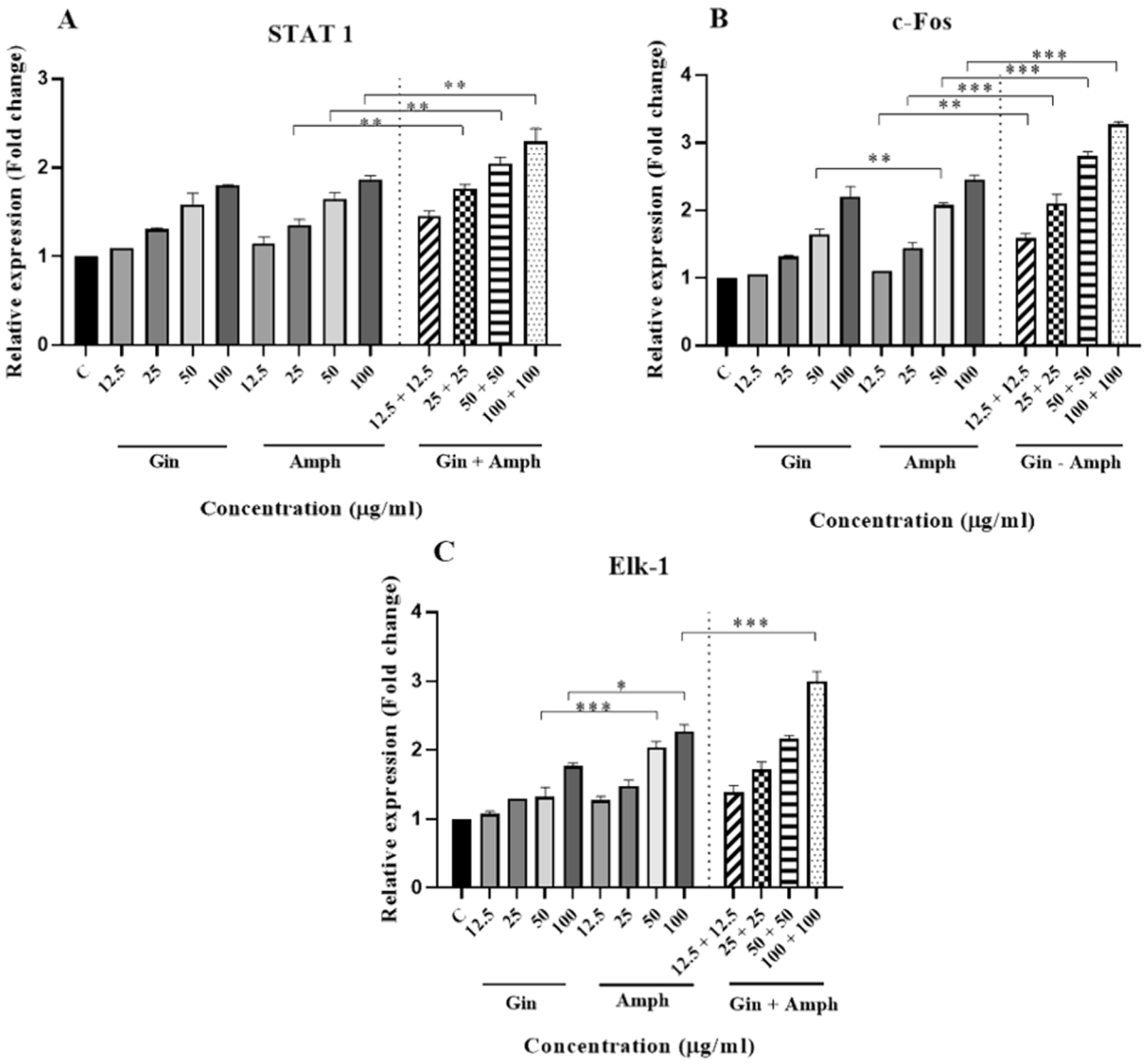

Fig. 6. The gene expression of the Th1-related transcription factors in macrophages treated with different concentrations of 6-gingerol (Gin), amphotericin B (Amph), and Gin + Amph compared to the untreated group. STAT1 (A), c-Fos (B), and Elk-1 (C). Error bars are SD (* $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$). Each test was conducted in triplicate.

medicine efficacy for severe cases in patients co-infected with HIV and for those whose treatment outcomes of monotherapy have consistently been insufficient [64]. These drugs act as the modulators of the host immune responses somewhat to the parasite, confirming a substitute approach for combination treatment. By measuring their effect on macrophage-derived cytokines (mostly IFN-γ, IL-12p40, TNF-α, and IL-10), the immunomodulatory potential of the drugs in leishmaniasis has been established [65].

The use of plant immunomodulators in synergy with conventional drugs such as amphotericin B may contain the valuable management of multiple molecular targets contributing to better therapeutic efficacy and diminished toxicity [66]. 6-Gingerol is probably an essential and unique multifunctional natural constituent known as biological response modifiers [67].

Here, we monitored the gene expression of the c-Fos, which is associated with the macrophage activation and Th1 response. The c-Fos gene is generally among the first to be expressed and referred to as an immediate-early gene, inducible by diverse agents. The c-Fos gene is contributed to the signal transduction cascade connecting extracellular impetuses to intracellular actions in response to various stimuli such as 6-gingerol [68]. To generate c-Fos, a protein programmed by the c-Fos gene, Elk1 must be phosphorylated by MAPKs [69]. MAPKs are the last effectors of signal transduction trails that initiate at the plasma membrane [70].

On the other hand, susceptibility to *L. major*-related CL is directly related to Th2 progression and production of relevant cytokines, including IL-4, IL-10, IL-13, and TGF-β, contributing to leishmanial replication, persistence, and subsequent immunopathological effects [55]. We also monitored these genes suppressing macrophage stimulation and maturation, leading to the restriction of intra-macrophage amastigotes in *L. major* infection [71]. These cytokines are directly involved with susceptibility to CL and persistence of parasites in the infection site, as indicated in this study [72]. Furthermore, IL-4 limits the production of the Th1 subset through downregulation of IL-12p40 [55,57]. TGF-β and IL-10 cytokines are pleiotropic cytokines with an immunoregulatory function that inhibit the Th1 response against the intracellular parasites by inactivating macrophages down-regulation of IFN-γ, iNOS, and TFN-α[73]. These cytokines also suppress innate and adaptive cell responses by inhibiting inflammatory cells' function and activating Treg cells' function (5,8).

Here, we observed a direct association between IL and 12p40 and IFN-γ, IL-12p40 and iNOS, and Elk1 and STAT-1. Conversely, this

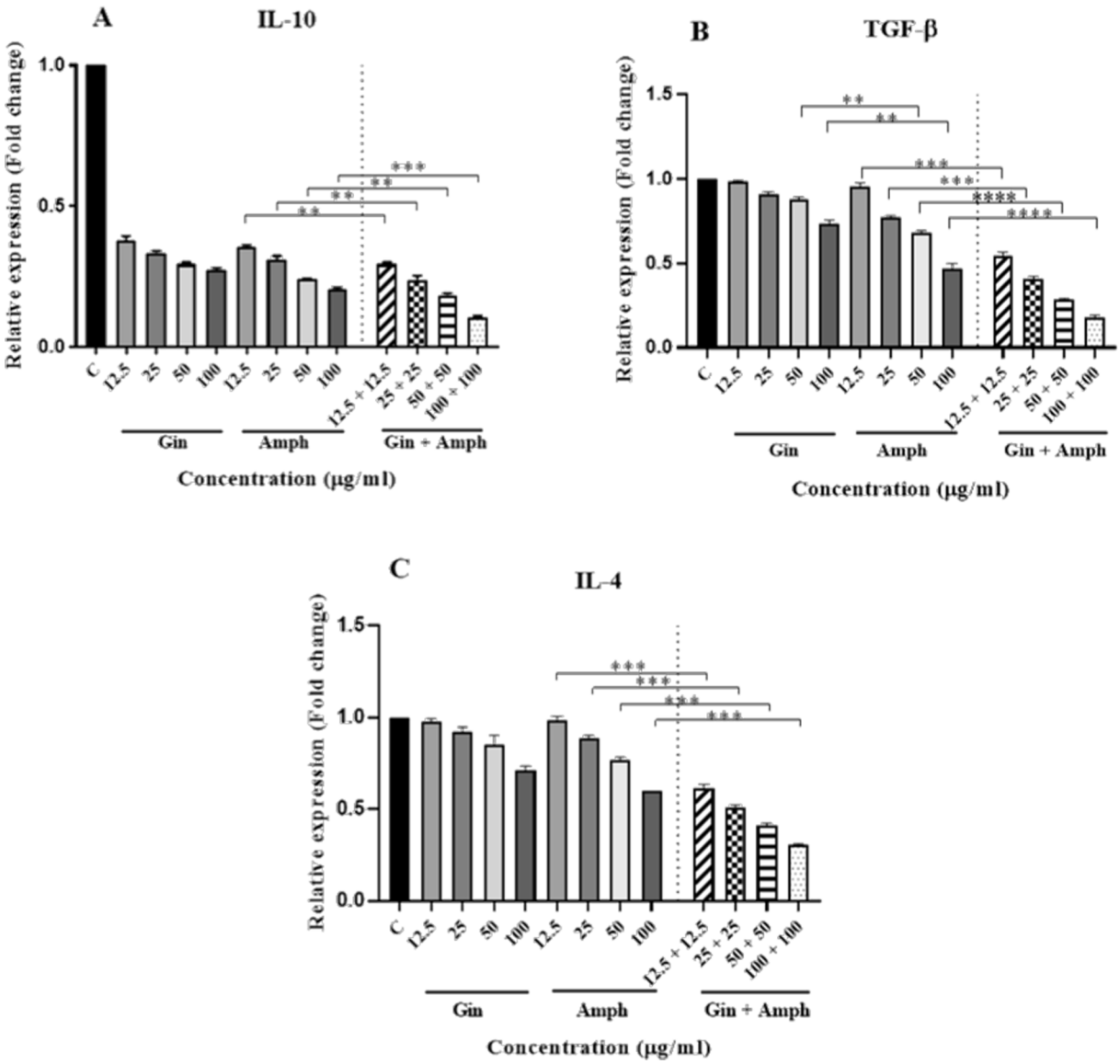

Fig. 7. The gene expression of Th2-related cytokines in macrophages treated with different concentrations of 6-gingerol (Gin), amphotericin B (Amph), and Gin + Amph compared to the untreated group. IL-10 (A), IL-4 (B), and TGF-β (C). Error bars are SD (**$p < 0.01$, ***$p < 0.001$ and ****$p < 0.0001$). Each test was performed in triplicate.

correlation was predictably reverse between IL and 4 and IFN-γ, and TNF-α and TGF-β. Overall, in this study, 8 out of the 13 significant expressed genes directly or indirectly are associated with the Th1 cell subsets led to the elimination of the intracellular amastigote stage. Gene expression profiling in the experimental model of CL has specified valuable evidence to understand the dynamic molecular pathways in the infected cells to discover new diagnostic/therapeutic targets. The cytokine's total gene expression contributing to the Th1 phenotype was significantly enhanced after treatment with gingerol or amphotericin B, but their effects were substantially enhanced when a combination of 6-gingerol and amphotericin B was used.

Similarly, we have indicated the antioxidant activity of 6-gingerol, which might act as a probable mechanism of action against CL. Confidently, the results proved that 6-gingerol is a multipotent bioactive component and possesses a high antioxidant activity level superior to that of standard (DPPH). 6-Gingerol could activate macrophages to exert a leishmanicidal effect through their enzymatic and oxidative metabolites. Antioxidants play a synchronized role in combination with cytokines while regulating the microenvironment they function [74]. Several naturally prevailing compounds in various plant products possess multipotent activities. These multifunctional antioxidants play a crucial role against diseases such as leishmaniasis. Therefore, discovering multidimensional natural complexes instead of solitary ones targeting fragments would be more beneficial [75].

Likewise, we studied programmed cell death (PCD), an unknown phenotypic and physiological manifestation in *Leishmania* species. For the lack of knowledge regarding the apoptotic process, proteins might play a pivotal role in survival and cell death under the pressure of the drug [76,77]. Pentavalent antimonial and miltefosine can activate different PCD paths in *Leishmania*. Foucher et al. [78] have revealed that amphotericin B and miltefosine triggered different morphological phenotypes of *Leishmania*, underlined by the lack of cell reduction. During apoptotic events, the balance of cytoplasmic bilayers is lost, and phosphatidylserine (PS) is exposed at the outer surface [79,80]. The apoptotic potential of 6-gingerol alone was observed through the cell signaling of PS as the cell membrane's fundamental component, but the effect was more profound when combined with amphotericin B. In the present study, 6-gingerol and amphotericin B strongly triggered the extrinsic trends of biological and molecular reactions and activated cell surface receptors as documented by PS externalization involved with

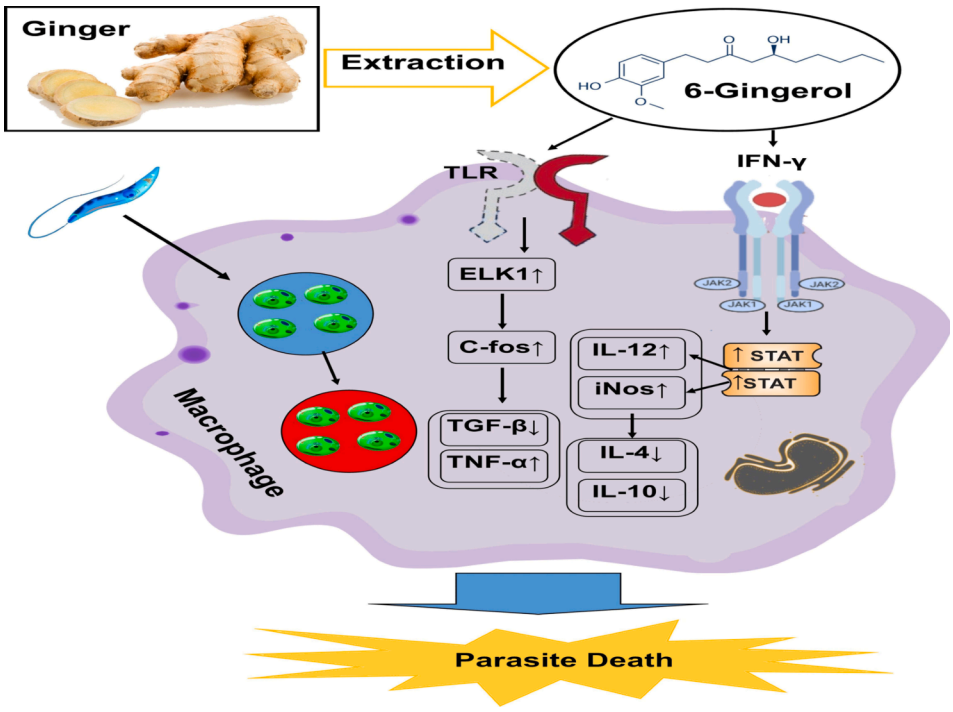

Fig. 8. Illustration of the gene expression of Th1/Th2-related parameters inducing by 6-gingerol combined with amphotericin B. Overall, Th1-relevant cytokines are up-regulated while Th2-associated cytokines are down-regulated.

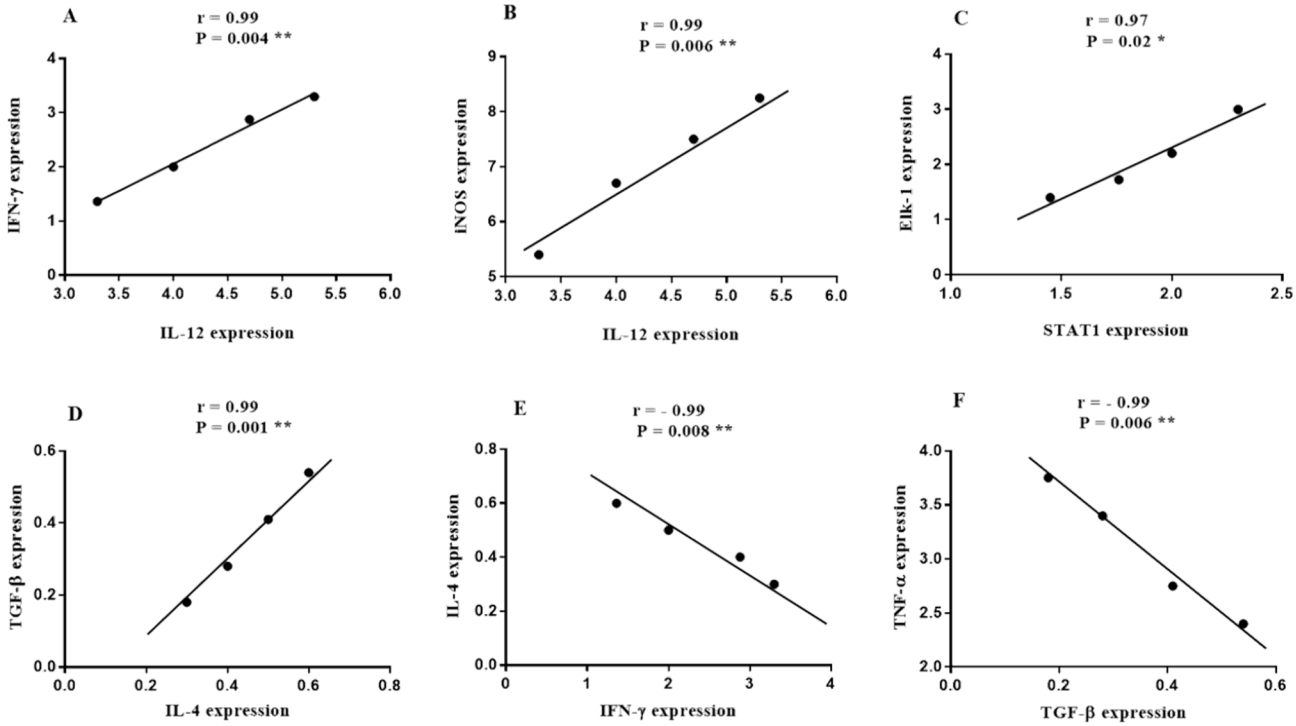

Fig. 9. Correlation level of cytokines expression in macrophages treated with different concentrations of Gin plus Amph. Positive (A, B, C and D) and negative (E and F) linear correlation were identified (* $p < 0.05$, ** $p < 0.01$).

apoptosis.

Many lines of evidence indicate that numerous plant constituents comprise immunomodulatory potentials [81], thus representing a striking substitute or complement of standard chemotherapy for leishmaniasis. Inclusive reviews have lately documented an extensive choice of antileishmanial alternatives from medicinal drugs [82], but less evidence is available on the modes of action of drugs against intracellular amastigotes. In the current study, we have confirmed substantial immunomodulatory, antioxidant, and apoptotic effects for 6-gingerol *in vitro* assays and significant therapeutic response in BALB/c mice to eliminate the parasite and cure CL lesions.

This investigation showed that 6-gingerol significantly mitigates the

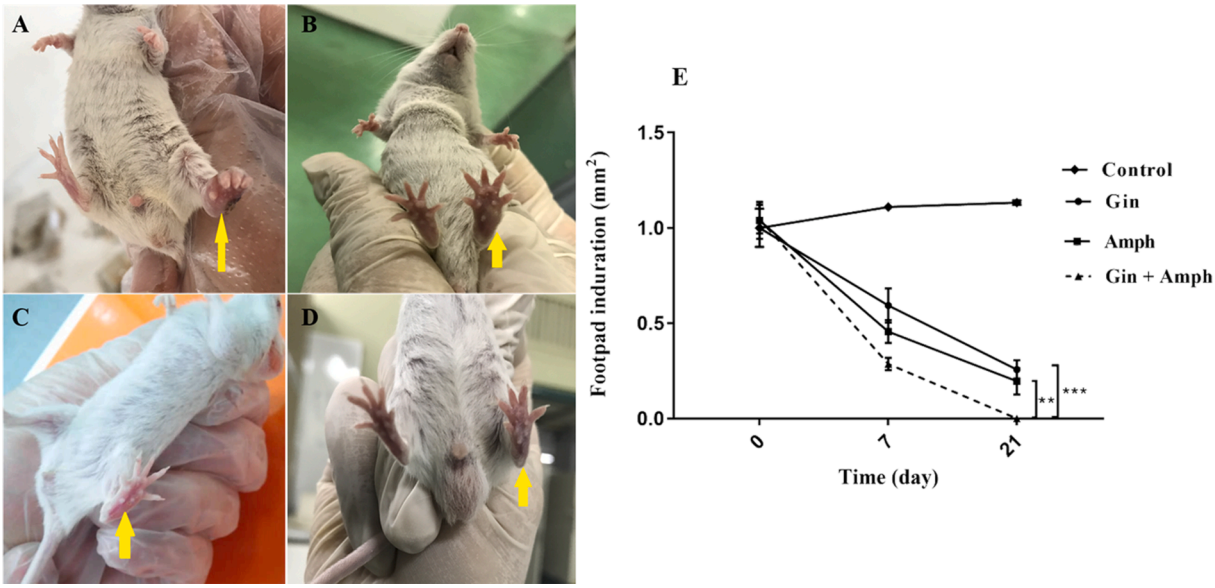

Fig. 10. Evaluation of the BALB/c mice footpad lesion in the untreated control. (A) 6-Gingerol, (B) Amphotericin B (positive control), (C) and 6-gingerol plus amphotericin B, (D) in the 3rd week after treatment. The healing process in the left footpad is detectable in the combination group (D) compared to each group alone, as shown by the arrow. The combination group significantly decreased the footpad induration compared to 6-gingerol or amphotericin alone (Fig. 11E). Error bars are SD (**$p < 0.01$ and ***$p < 0.001$).

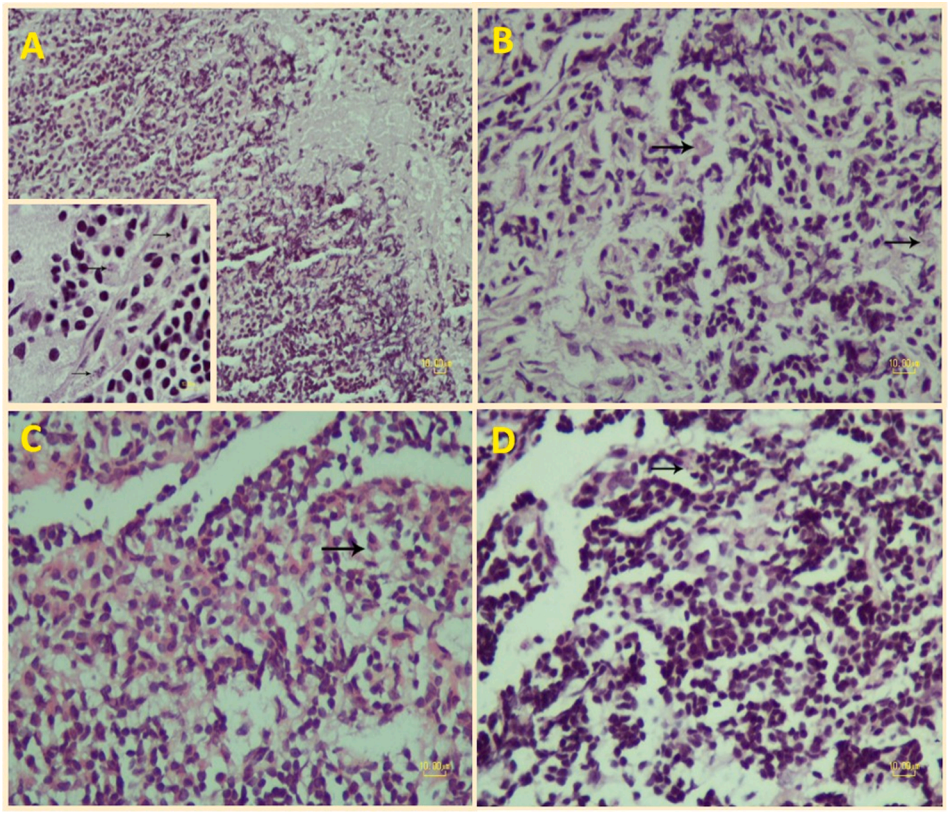

Fig. 11. Histopathological sections of popliteal lymph nodes in different treatment groups of BALB/c mice. A) Negative control group, structures of the popliteal lymph nodes are disorganized with necrosis, H&E, Bar = 10 μm, infiltration of histiocytes containing a moderate number of Leishman bodies (arrows) in the lymph node medulla, H&E, Bar = 40 μm, B) Amphotericin B group) In this photomicrograph, sinus histiocytosis and a low number of parasitic load (arrows) are seen, H&E, Bar = 10 μm, C) 6-Gingerol group) Infiltration of mononuclear cells and histiocytes with low parasitic load (arrow) are seen, H&E, Bar = 10 μm, D) 6-Gingerol combined with the amphotericin B group) Minimal sinus histiocytosis with a negligible number of Leishman bodies (arrow) is seen, H&E, Bar = 10 μm. The organisms were quantified based on the Ridley parasitic index [15].

parasite load in BALB/c mice within three weeks. Furthermore, the CL lesions from BALB/c mice that received 6-gingerol combined with amphotericin B exhibited fewer parasites than the lesions from mice received 6-gingerol or amphotericin B alone as measured by the standard grading system of Ridley [44]. The standard therapy for CL is generally 28 days abroad, and this schedule could achieve complete. These data represent that the parasite burden may directly affect cytotoxic cells' recruitment, affecting how the host responds to the treatment regimen. Therefore, 6-gingerol combined with amphotericin B possesses a powerful ability to cure CL in BALB/c mice and can be used as a model drug in a future clinical setting.

Additionally, the composition of the infiltrated immune cells might have a direct role in the resolution of the lesion. They compressed and surrounded the Leishman bodies engulfed macrophages [83]. B cells

Table 7
Average parasite density and score (grade) in 5 sections of lymph nodes corresponding to BALB/c mice.

Groups	No. of amastigotes/5 sections
Negative control	5, 5, 6, 5, 5
Amphotericin B	3, 4, 3, 3, 3
6-Gingerol	2, 2, 2, 3, 2
6-Gingerol plus amphotericin B	0, 0, 1, 0, 0

More than 100,000 parasites per standard section (6), 10,100–100,000 parasites per standard section (5), 1001–10,000 parasites per standard section (4), 101–1000 amastigotes per standard section (3), 11–100 amastigotes per standard section (2), 1–10 amastigotes per standard section (1), No amastigotes (0) [40].

were not so increased in numbers. These histopathological findings confirmed that this therapy had the excellent benefit of decreasing Leishman bodies in their reservoir histiocytes and has activated cellular immunity to compensate multiplication of Leishman bodies within the parasitophorous vacuoles of the macrophages.

IHC profiling in parasitic diseases is another fundamental instrument to understand the pathogenesis of CL induced by a drug [8]. The parasite burden and the determination of the phenotype of the infiltrated leukocytes in the leishmanial lesions are valuable biomarkers for monitoring the therapeutic targets. Here, we showed a better immune response in the involved lymph node as decreasing and degenerative macrophages and increasing lymphocytes as mostly $CD4^+$ T more than $CD8^+$ T lymphocytes.

Amphotericin B combined with γ-cyclodextrin (γ-CD) have been used as a topical formulation *in vivo* and also *in vitro* assays against different fungal infections and CL species in the New World. γ- CD was chosen to solubilize amphotericin B. γ-CD has demonstrated a synergistic effect with amphotericin B and this novel formulations based on amphotericin B–CD complex have shown higher antifungal effect relative to amphotericin B NeoSensitabs disks, amphotericin B dissolved in dimethyl sulfoxide (DMSO) and clotrimazole cream, respectively. Furthermore, amphotericin B–CD methylcellulose gel has significantly exhibited greater inhibition action on biofilm formation and superior fungicidal activity on biofilm cells than amphotericin B dissolved in DMSO. Also, amphotericin B–CD gel displayed high *in vitro* anti-leishmanial effectiveness with broader therapy profile and *in vivo* effect against three different tegumentary CL species. The outcome represented the feasibility and safety of topical amphotericin B preparation and diverse fungal and CL parasitic species. They used golden hamsters for *in vivo* leishmanicidal activity assay in an experimental cutaneous model [84].

In conclusion, 6-gingerol combined with amphotericin B synergistically exerted anti-leishmanial activity *in vitro* and *in vivo*, enhanced apoptosis in the parasite, and potentiated the leishmanicidal activity of macrophages. This combination also supported IFN-γ-mediated signaling, reduced parasite burden in the infected mice, and modulated Th1- and Th2-related phenotypes. Furthermore, the above mixture improved the histopathological changes in the BALB/c mice infected with *L. major* and modulated the leukocyte infiltration into the lesions. Finally, 6-gingerol exhibited a capacity for binding to IFN-γ, displayed potent antioxidative activity, and had no cytotoxic effect on mammalian macrophages. These broad mechanistic insights provide a coherent basis

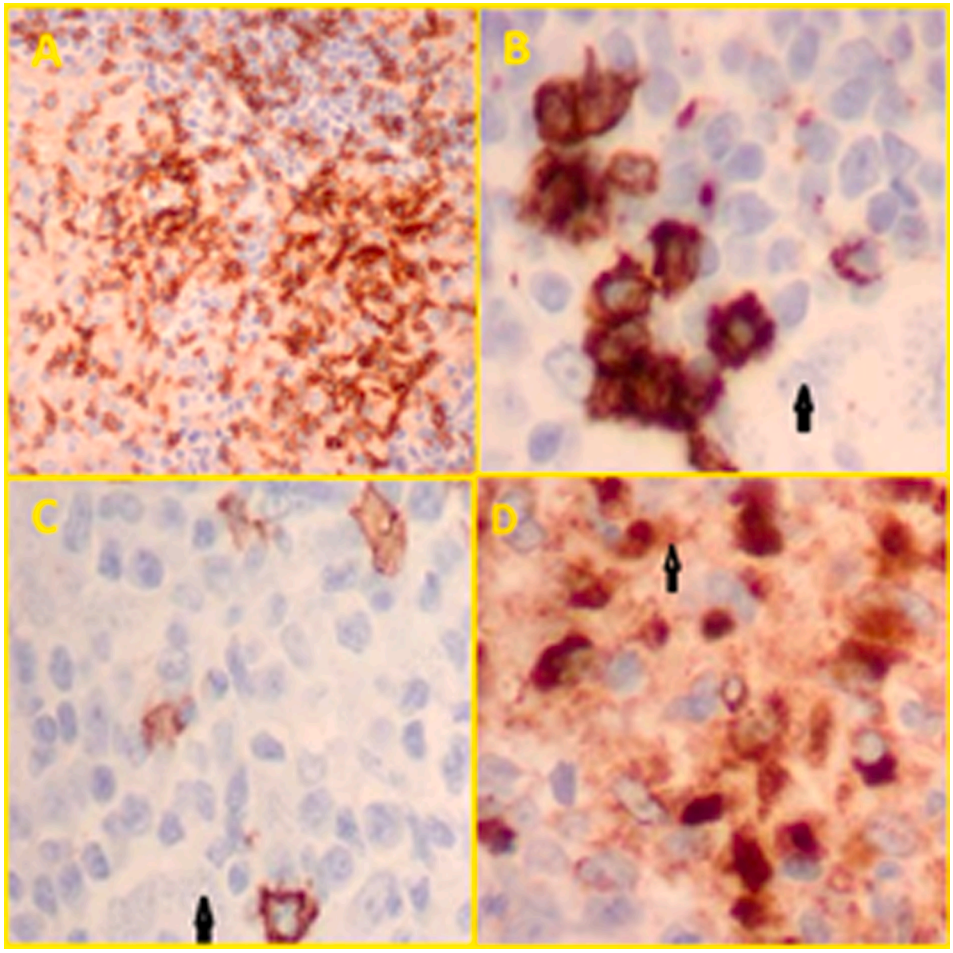

Fig. 12. Immunohistochemical findings in sections of popliteal lymph nodes of BALB/c mice treated with a combination of 6-gingerol and amphotericin B. A) Cluster of $CD4^+$ T lymphocytes which compressed the macrophages, B) Clusters of $CD8^+$ T cells around the engulfed intracytoplasmic Leishman bodies of macrophage, C) Rare scattered $CD20^+$ B lymphocytes and a macrophage with intracytoplasmic Leishman bodies, D) $CD68^+$ macrophages with degenerative changes.

for further clinical study using 6-gingerol as a potential drug candidate for CL.

Declaration of Competing Interest

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

Acknowledgments

We would like to thank all the personnel of Leishmaniasis Research Center for their help in conducting this study. We also appreciate Dr. Ali Afgar, Department of Medical Parasitology and Mycology, School of Medicine, Kerman University of Medical Sciences, for assisting in molecular docking.

Funding

This study was supported by National Institute for Medical Research Development (NIMAD), Grant No 971413.

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