

Antileishmanial Activity of Niosomal Combination Forms of Tioxolone along with Benzoxonium Chloride against *Leishmania tropica*

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Abstract: In this study, we carried out extensive in vitro studies on various concentrations of tioxolone along with benzoxonium chloride and their niosomal forms against *Leishmania tropica*. Niosomes were prepared by the hydration method and were evaluated for morphology, size, release study, and encapsulation efficiency. This study measured leishmanicidal activity against promastigote and amastigote, apoptosis and gene expression levels of free solution and niosomal-encapsulated tioxolone along with benzoxonium chloride. Span/Tween 60 niosome had good physical stability and high encapsulation efficiency (more than 97%). The release profile of the entrapped compound showed that a gradual release rate. The combination of niosomal forms on promastigote and amastigote were more effective than glucantime. Also, the niosomal form of this compound was significantly less toxic than glucantime ($P \leq 0.05$). The flowcytometric analysis on niosomal form of drugs showed that higher number of early apoptotic event as the principal mode of action (89.13% in 200 µg/ml). Also, the niosomal compound increased the expression level of IL-12 and metacaspase genes and decreased the expression level of the IL-10 gene, which further confirming the immunomodulatory role as the mechanism of action. We observed the synergistic effects of these 2 drugs that induced the apoptotic pathways and also up regulation of an immunomodulatory role against as the main mode of action. Also, niosomal form of this combination was safe and demonstrated strong anti-leishmaniasis effects highlights further therapeutic approaches against anthroponotic cutaneous leishmaniasis in future planning.

Key words: *Leishmania tropica*, tioxolone, benzoxonium chloride, niosome

INTRODUCTION

Leishmaniasis, the third most important vector-borne diseases, caused by obligate intra-macrophage protozoa that is endemic in the tropics and sub-tropics area [1]. It is a complex disease with diverse clinical forms and among which, the cutaneous presentation, is the most common [2]. Conventional chemotherapy is the only effective measure against this disease. The first-choice compounds for the treatment of leishmaniasis includes pentavalent antimonials such as sodium sti-

bogluconate (Pentostam), and meglumine antimoniate (Glucantime®). Despite the recent advances, these chemotherapeutic measures have a number of important disadvantages such as severe side effects, the emergence of resistance, daily parenteral or weekly intralesional administration, and high cost [3]. Also, the advent of resistance and chronicity occurs in anthroponotic cutaneous leishmaniasis (ACL) due to *Leishmania tropica* more frequently than other *Leishmania* species. Therefore, development of an effective medication against the disease is very important [4]. Combination therapy can also have many benefits such as synergistic activity, reduced dosage and duration of treatment, thereby increasing compliance; reduction of the cost and adverse effects of therapy, as well as reducing the risk of resistance.

Currently, numerous investigations have been carried out to find a low-risk, effective, inexpensive and easy-to-use drug.

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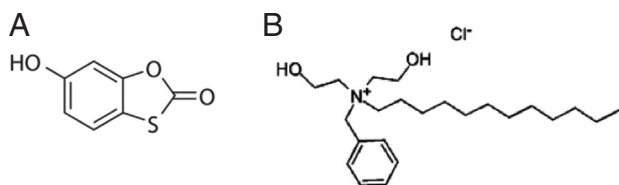


Fig. 1. Chemical structures of (A) 6-hydroxy-1, 3-benzoxathol-2-one (tioxolone) and (B) benzylododecylbis (2-hydroxyetyl) ammonium chloride (benzoxonium chloride).

Meanwhile, the new pharmaceutical formulations such as liposome and niosome, especially with topical application, appear to be promising because surrounding tissues are not affected by the drug and loss of drug does not happen due to localization, leading to enhance maximum efficacy of the medication. Niosomes are the best carriers and are vesicles composed of non-ionic surfactants, which are biodegradable, more stable and inexpensive [5].

In previous study we reported the therapeutic effect of topical application that was named Thio-Ben. This drug was a mixture of tioxolone and benzoxonium chloride. Their clinical trial showed that the topical use of Thio-Ben was as effective as the intralesional treatment with glucantime [6]. Tioxolone (C₇H₄O₃S) (Fig. 1A), have been used for local treatment of acne and psoriasis vulgaris and also known as anti-fungal, anti-bacterial, anti-inflammatory and anti-tumorigenic effects [7,8]. Also, benzoxonium chloride (C₂₃H₄₂ClNO₂) (Fig. 1B) belongs to quaternary ammonium compounds of anti-infective agents that are used as anti-septic drugs and for disinfecting the mucus membrane, skin surface, and medical instruments. Benzoxonium chloride is also effective against bacteria, certain protozoa, yeasts and non-spore forming organisms [9]. Since the niosomal formulation of combine these 2 drugs may potentially be more effective on *L. tropica*, therefore, in this study, we carried out extensive studies in in vitro models using various concentrations of tioxolone along with benzoxonium chloride and its niosomal forms.

MATERIALS AND METHODS

Preparation and characterization of niosomes

Niosomal vesicles were prepared by the hydration method as reported previously [10]. Briefly, the nonionic surfactants (Span 20, 40, 60, 80 and Tween 20, 40, 60, 80) and cholesterol were dissolved in chloroform and mixed at different molar ratios (7:3, 6:4, and 8:2) in a round-bottom flask. Tioxolone (5,000

µg/ml) was dissolved in 100 ml chloroform and 5 ml added in the flasks. The solvent was evaporated using a rotary evaporator (Büchi, Flawil, Switzerland) at 180 rpm and 70°C for 15 min and a thin layer of lipid film formed on the wall of the flask.

The lipid film was hydrated with 5 ml of 100 ml deionized water containing 3,000 µg/ml of benzoxonium chloride to the flask at 70°C for 30 min. The resulting niosomal suspensions were maintained over night at room temperature for further experimentations. The morphology of vesicles was observed using light microscopy (Zeiss, Oberkochen, Germany). Size of vesicles was evaluated by measuring their dynamic light scattering with Master Sizer 2000 E (Malvern Instruments, Malvern, UK). Also, physical stability of niosomes was determined by size variations over 3 days, 1, 3, and 6 months after production.

Release profiles of entrapped drugs were determined by dialysis method [11] in selected formulations using the dialysis bag (cellulose acetate membrane, Sigma-Aldrich, Steinheim, Germany) at 37°C for certain time intervals (0, 15, 30, 60, 90, 120, 150, 180, 210, 240 min). The drugs concentrations in the samples were measured using the spectrophotometric method at 287.9 nm for tioxolone and at 208 nm for benzoxonium chloride. Also, encapsulation efficiency was performed by dialysis method and calculated the entrapped drug [12] as per the following equation:

$$\% \text{ Encapsulation efficiency} = \frac{\text{Total drug} - \text{free non entrapped drug}}{\text{Total drug}} \times 100$$

Cells and parasite

Standard strain of *L. tropica* (MHOM/IR/2002/Mash2) was prepared from the Leishmaniasis Research Center (Kerman, Iran) and cultured in RPMI-1640, 1 µl penicillin-streptomycin (10,000 IU/ml), 15% heat-inactivated fetal bovine serum (FBS) and incubated at 25°C ± 1°C. Murine macrophage cells J774 A.1 ATCC®TIB-67™ were purchased from the Pasteur Institute of Iran and cultured in DMEM, 1 µl penicillin-streptomycin (10,000 IU/ml) and 15% inactivated FBS at 37°C and 5% CO₂.

The viability rate of macrophages and serial dilutions

At first, the cytotoxicity of the tioxolone (T) along with benzoxonium chloride (B) and their selected niosomal forms (NT and NB) were carried out separately by MTT assay on J774 A.1. Then once the concentrations of the T drug were fixed and the concentration of B was increased. Again, we kept the concentrations of B constant and increased the concentration of T

drug. In the niosomal combination of NT and NB, at first, dilgence was done based on NT and then serial dilutions were based on NB.

Antipromastigote assay

L. tropica (10^5 parasites/ml) in the logarithmic growth phase were incubated in RPMI-1640 medium supplemented with 10% FBS in the presence of several concentrations of tioxolone along with benzoxonium chloride and its niosomal forms as triplicate in 96-well plates at $25^\circ\text{C} \pm 1^\circ\text{C}$ for 48 hr. Simultaneously, meglumine antimoniate (glucantime) and medium with no drugs were used as positive and negative control, respectively. Leishmanicidal activity of tioxolone along with benzoxonium chloride and niosomal forms of this combination at the promastigote stage of *L. tropica* were measured by MTT analysis.

Antiamastigote assay

Attached macrophages were infected with *L. tropica* promastigotes (stationary growth phase) at a parasite/macrophage ratio of 10:1 and incubated at 37°C and 5% CO_2 for 24 hr. Then, the macrophages containing parasites were treated with serial dilutions of all drugs and control in triplicates, were incubated at 37°C and 5% CO_2 for 48 hr. Cells were then fixed with 100% methanol fixative, stained with Wright Giemsa for 15 min. The average number of amastigotes in 100 macrophages was determined by examining 3 slides for each treatment by direct microscopy and the results were compared to controls. The survival index was determined by multiplying the number of infected cells by the mean number of amastigotes per cell and the results were used to establish a proportion in relation to the control.

Determination of apoptosis

Apoptotic and necrotic cell events were studied by PE Annexin V and 7-Amino Actinomycin D (7-AAD) with PE Annexin V Apoptosis Detection Kit I (BD Pharmingen™, BD Biosciences, San Jose, California, USA). The *L. tropica* promastigotes (1×10^6) were treated with the desired concentrations of selected combination drugs and incubated at 25°C for 48 hr. Then, promastigotes were washed in cold phosphate-buffered saline (PBS) twice, suspended in 1X binding buffer and were added 5 μl of PE Annexin V and 5 μl 7-AAD in the dark for 15 min. The samples were analyzed by flowcytometry (BD FACS-Calibur™, San Jose, California, USA).

RNA isolation and analysis

RNA was extracted using the RNeasy Mini Kit (Qiagen, Chatsworth, California, USA) according to the manufacturer's instructions at various concentrations of effective drug combination and untreated control. The cDNA was synthesized by a first-strand cDNA Synthesis Kit (Takara Bio, Inc., Shiga, Japan). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as reference for gene expression of IL-12 and IL-10 in murine macrophage cells (J774 A. 1) and RPS18 Ribosomal protein (S18) for gene expression of metacaspase in promastigote and they were detected by quantitative real-time PCR (q-PCR) assay [13,14]. The ΔCT was measured by means of the following formula: [$\Delta\text{CT} = \text{CT}(\text{target}) - \text{CT}(\text{reference})$]. Gene expression level was specified by the $2^{-\Delta\text{CT}}$ methods. Moreover, fold increase (FI) was measured through the comparative threshold method ($2^{-\Delta\Delta\text{CT}}$).

Statistical analyses

Data were analyzed by one-way analysis of variance (ANOVA), and differences between groups were assessed using Bonferroni test in the SPSS software version 20 (Chicago, Illinois, USA). Results were considered significant at $P \leq 0.05$. The graphs were designed using GRAPHPAD PRISM 6 (GraphPad Software Inc, San Diego, California, USA).

RESULTS

Characterization of niosomes

The morphology of prepared niosomes and their particle

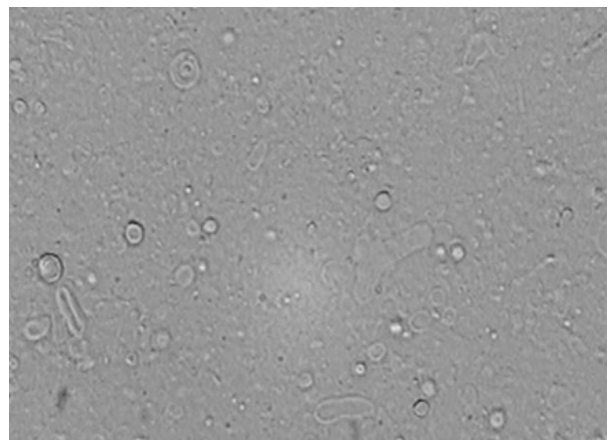


Fig. 2. The light microscopic picture of niosomal forms of tioxolone along with benzoxonium chloride ($\times 100$ magnification) prepared by film hydration method.

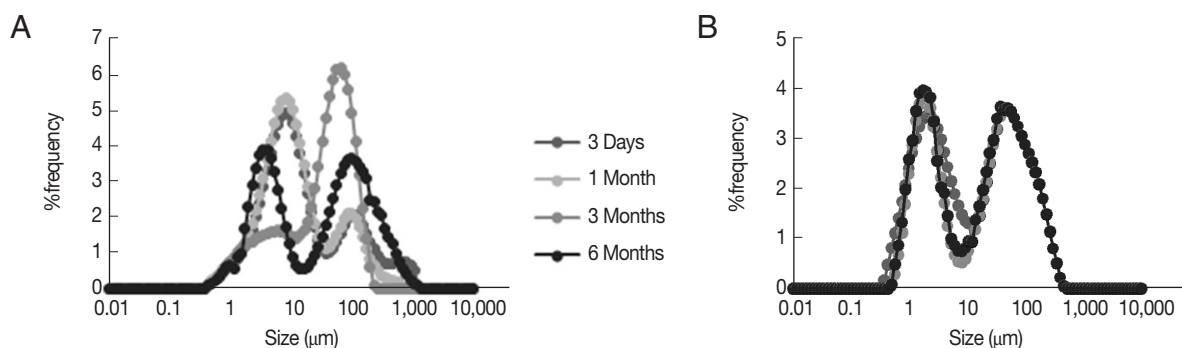


Fig. 3. The particle size distribution graph by frequency of (A) Span Tween 40 (6:4 m.r., NTB₁) and (B) Span Tween 60 (6:4 m.r., NTB₂) at 3 days, 1, 3, and 6 months.

Table 1. Encapsulation efficiency percentage (%EE) of niosomal tioxolone along with benzoxonium chloride formulation

Active ingredient	λ max (nm)	Absorbance	Concentration (μ g/ml)	Amount (Dialysate solution)	Total (μ g/ml)	%EE
Tioxolone	278	0.15	7.04	105.6	5,000	97.88
Benzoxonium chloride	208	0.17	5.26	78.9	3,000	97.37

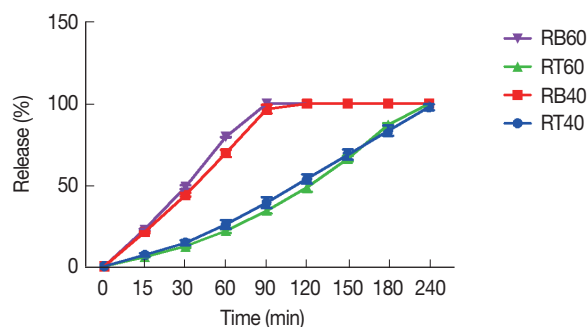


Fig. 4. The released amount of niosomal of tioxolone (% RT) and benzoxonium chloride (% RB) from the selected formulations at different time intervals (mean \pm SD, n=3).

size distribution showed that, the best formulations were Span/Tween 40 (ST40, 6:4 molar ratio, NTB₁) and Span/Tween 60 (ST60, 6:4 m.r., NTB₂) (Figs. 2,3). Size analysis of 2 formulations showed 2 log-normal particle size distribution curves after 3 days but only NTB₂ had good physical stability, as depicted in their size distribution curves during 1, 3, and 6 months (Fig. 3). High encapsulation efficiency (more than 97%) was calculated for NTB₁ and NTB₂ (Table 1). The release profile of niosomal forms of entrapped materials showed that the release rate of tioxolone was a mild gradient but benzoxonium chloride was completely released at 90 min. Both formulations (NTB₁ and NTB₂) showed a similar release rate (Fig. 4).

The viability rate of macrophages

The viability rates of macrophages after treatment with com-

bination of tioxolone (T) along with benzoxonium chloride (B) in comparison with untreated control were showed a significant difference ($P < 0.001$) (Fig. 5A). Also, the viability rate of macrophages is reduced by increasing the concentration of each drug (T or B). Whereas, these rate in combinations of niosomes were higher than glucantime ($P \leq 0.05$) (Fig. 5B). The viability rate of macrophages at 12.5, 25, 50, 100, and 150 μ g/ml concentrations of glucantime were 87.46 ± 0.15 , 77.96 ± 0.35 , 70.50 ± 0.50 , 68.20 ± 0.40 , and 67.00 ± 0.20 , respectively.

Antileishmanicidal activity on promastigote

The percentage of inhibition rate of *L. tropica* promastigotes treated with various concentrations of tioxolone (T) along with benzoxonium chloride (B) in comparison with T alone showed a dose-dependent response, as presented in Fig. 6A. Also, the inhibition rate of promastigotes treated with various concentrations (12.5, 25, 50, 100, and 150 μ g/ml) of glucantime were 19.86 ± 1.27 , 24.03 ± 0.35 , 29.76 ± 1.16 , 35.80 ± 0.40 , and 39.66 ± 0.41 , respectively, that in comparison with these rates in tioxolone along with benzoxonium chloride were significantly lower ($P \leq 0.05$).

The inhibitory rates of *L. tropica* promastigotes treated with various concentrations of the combinations of niosomes compared with glucantime, as presented in Fig. 6B, which indicates that their inhibitory rates were significantly higher than glucantime ($P \leq 0.05$).

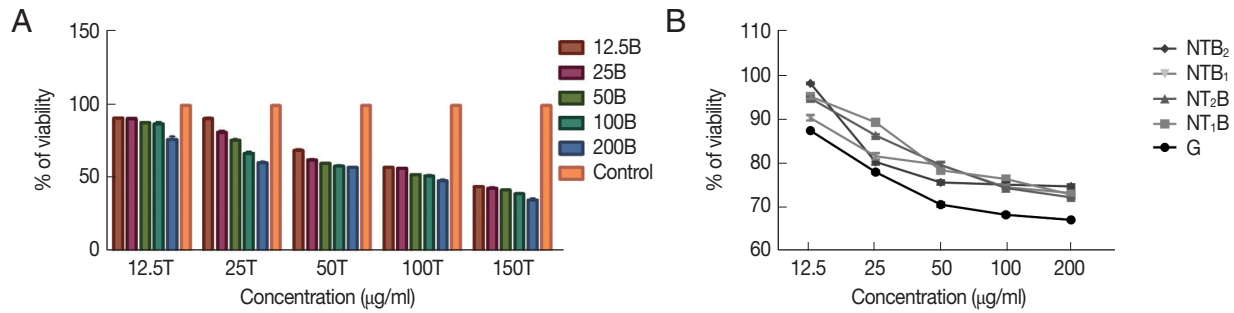


Fig. 5. The viability of J774 A.1 cells in the presence of various concentrations of combination of (A) tioxolone (T) along with benzoxonium chloride (B) in comparison with untreated control; (B) niosomes (NTB) in comparison with glucantime (G) after 48 hr incubation. Bars represent the mean ± standard deviation of viability rates (NT₁B and NT₂B: serial dilutions were based on NB; NTB₁ and NTB₂: serial dilutions were based on NT).

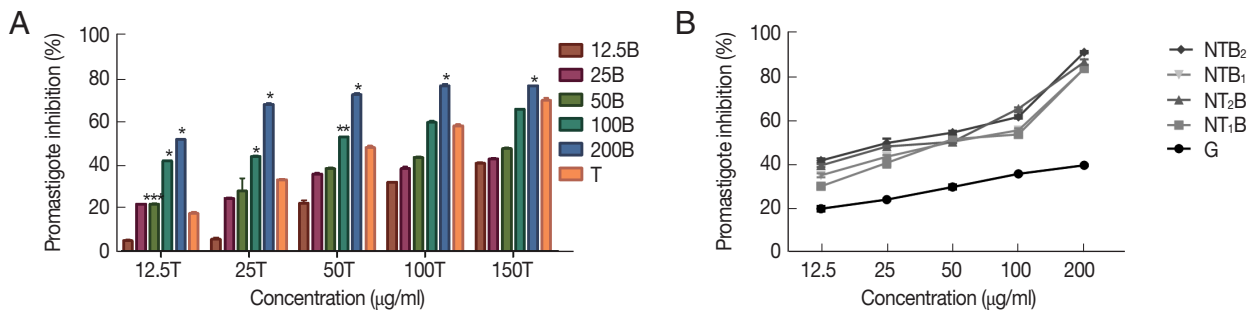


Fig. 6. The inhibitory rates of *L. tropica* promastigotes treated with various concentrations of (A) tioxolone (T) along with benzoxonium chloride (B) in comparison with the T alone; (B) the combinations of niosomes in comparison with glucantime (G) after 48 hr incubation. Bars represent the mean ± SD of inhibitory rates (**P* < 0.001, ***P* < 0.01, ****P* < 0.05 and NT₁B and NT₂B: serial dilutions were based on NB; NTB₁ and NTB₂: serial dilutions were based on NT).

Table 2. Comparative evaluation of the effect of tioxolone (T, 150, 100, 50, 25, 12.5 µg/ml) combined with various concentrations of benzoxonium chloride (B, 200, 100, 50, 25, 12.5 µg/ml) with untreated control on intramacrophage amastigotes of *L. tropica* after 48 hr incubation (*P* ≤ 0.05)

Concentration (µg/ml)	12.5B		25B		50B		100B		200B	
	No. of amastigotes	<i>P</i> -value	No. of amastigotes	<i>P</i> -value	No. of amastigotes	<i>P</i> -value	No. of amastigotes	<i>P</i> -value	No. of amastigotes	<i>P</i> -value
0.0 (Control)	60.71 ± 1.46	NR	60.71 ± 1.46	NR	60.71 ± 1.46	NR	60.71 ± 1.46	NR	60.71 ± 1.46	NR
12.5 T	43.70 ± 0.60	0.001	40.60 ± 0.70	0.001	36.20 ± 0.70	0.001	32.16 ± 0.70	0.001	29.26 ± 0.25	0.001
25 T	37.56 ± 0.55	0.001	35.50 ± 0.30	0.001	33.16 ± 0.40	0.001	29.30 ± 0.85	0.001	24.33 ± 0.65	0.001
50 T	32.33 ± 0.25	0.001	30.26 ± 0.60	0.001	27.40 ± 0.30	0.001	25.43 ± 0.35	0.001	22.10 ± 0.30	0.001
100 T	30.30 ± 0.50	0.001	29.40 ± 0.50	0.001	25.93 ± 0.45	0.001	22.70 ± 0.30	0.001	20.66 ± 0.32	0.001
150 T	29.90 ± 0.34	0.001	29.00 ± 0.50	0.001	23.70 ± 0.20	0.001	21.46 ± 0.41	0.001	19.33 ± 0.32	0.001

Data represent the mean value ± SD.
NR, Not related.

Antileishmanicidal activity on amastigote

The average number of amastigotes in 100 macrophages at various concentrations of T with B is shown in Table 1, which represents that with increasing concentration of each drug, the number of amastigotes was decreased. This combination of T150 µg/ml and B200 µg/ml showed the least number of

amastigotes (19.33 ± 0.34). Also, in niosomal formulations, with increasing concentration, the number of amastigotes were decreased, so that, the least number of amastigote (10.51 ± 0.43) belongs to the highest concentration of NTB₂ formulation (200 µg/ml).

Various concentrations of the drugs (T along with B or NTB)

Table 3. Comparative evaluation of various concentrations of the combination niosomes and glucantime with untreated control on intramacrophage amastigotes of *L. tropica* after 48 hr incubation ($P \leq 0.05$) (NT₁B and NT₂B: serial dilutions were based on NB; NTB₁ and NTB₂: serial dilutions were based on NT)

Concentration (µg/ml)	12.5		25		50		100		200	
Drugs	No. of amastigote	P-value	No. of amastigote	P-value	No. of amastigote	P-value	No. of amastigote	P-value	No. of amastigote	P-value
0.00 (Control)	60.71 ± 1.46	NR	60.71 ± 1.46	NR	60.71 ± 1.46	NR	60.71 ± 1.46	NR	60.71 ± 1.46	NR
Glucantime	54.20 ± 1.06	0.013	41.86 ± 1.02	0.001	34.46 ± 2.05	0.001	31.46 ± 1.91	0.001	22.96 ± 2.62	0.001
NBT ₁	34.60 ± 0.34	0.001	22.67 ± 0.57	0.001	19.19 ± 0.68	0.001	18.49 ± 0.34	0.001	14.17 ± 0.19	0.001
NBT ₂	33.44 ± 0.71	0.001	21.71 ± 0.75	0.001	19.81 ± 0.54	0.001	16.27 ± 1.14	0.001	10.92 ± 0.39	0.001
NTB ₁	35.55 ± 1.28	0.001	23.85 ± 0.68	0.001	23.29 ± 0.60	0.001	22.12 ± 0.89	0.001	12.26 ± 0.44	0.001
NTB ₂	25.55 ± 1.26	0.001	17.71 ± 0.58	0.001	16.07 ± 0.46	0.001	13.54 ± 1.12	0.001	10.51 ± 0.43	0.001

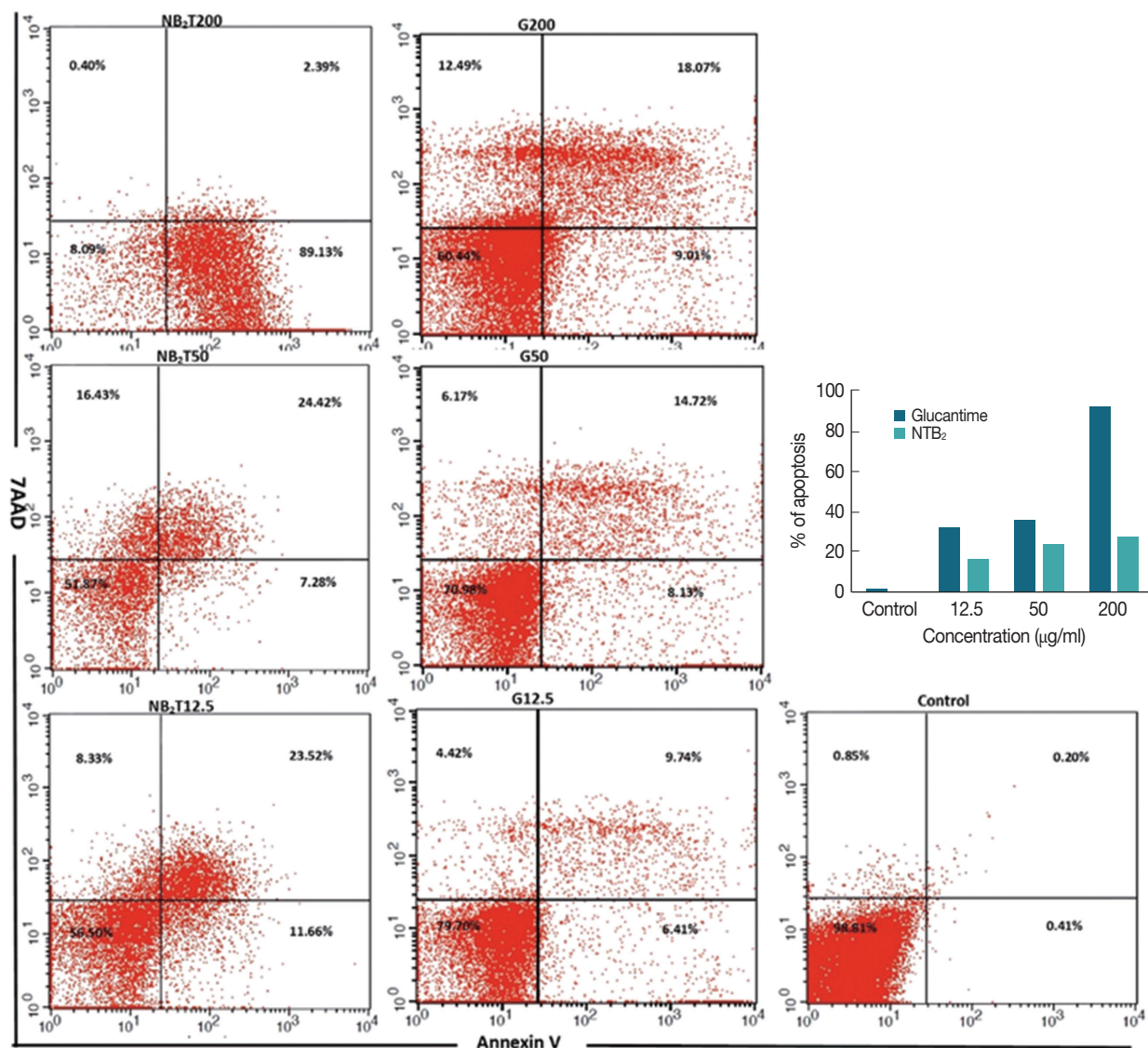


Fig. 7. The apoptotic and necrotic profiles of *Leishmania tropica* promastigotes treated with various concentrations of NTB₂ (serial dilutions were based on NT₂) compared with positive and untreated control.

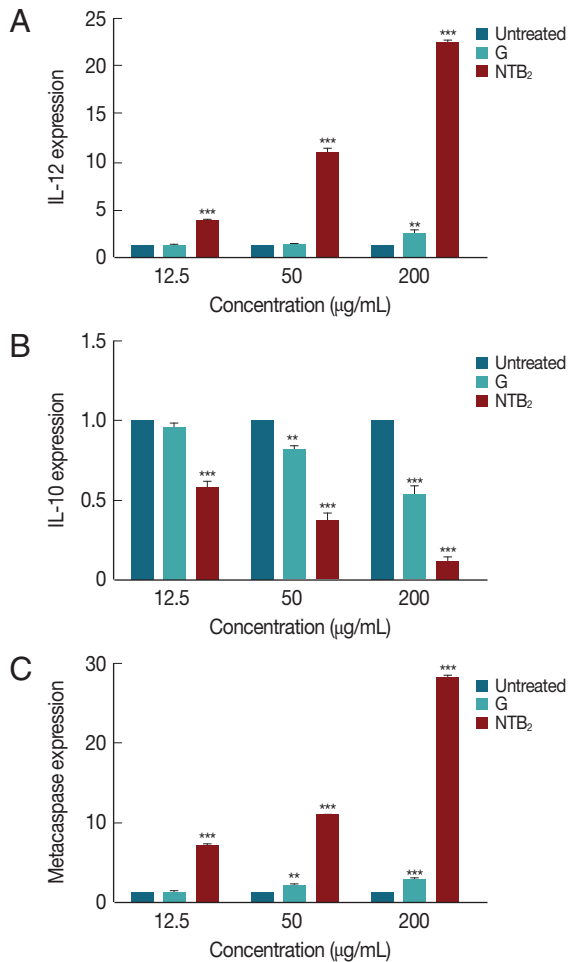


Fig. 8. Effect of different concentrations of NTB₂ and positive control (G) on genes expression of (A) IL-12, (B) IL-10, and (C) metacaspase of *L. tropica* (NTB₂ and G compared to untreated control; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

showed a significant reduction ($P < 0.001$) in the number of amastigotes compared to the untreated control group (Tables 2, 3). Also, the mean number of amastigotes at different concentrations of niosomal formulations was significantly ($P < 0.001$) less than that of glucantime (Table 3).

Apoptosis

The levels of apoptotic, necrotic, and viable cells at 3 different concentrations of the most effective combination of niosomal form (NTB₂) were determined and compared with the untreated and positive control (G) (Fig. 7). At the concentration of 200 µg/ml of niosomal combination of NTB₂ formulation the best effect on the promastigote and amastigote stages, with a high rate of apoptosis (89.13% early and 2.39% late apoptosis) was observed. However, the rates of induced apop-

toxis in the G₂₀₀ were 9.01% early and 18.07% late. Also, at the lower concentrations of this combination of niosome, a relatively high rate of apoptosis was observed (31.7% & 37.2% in NTB₂ 50 µg/ml and NTB₂ 12.5 µg/ml, respectively).

Gene expression

The genes expression level of IL-12, IL-10, and metacaspase in response to *L. tropica* was evaluated against various concentrations of combination niosome NTB₂ (Fig. 8). IL-12 expression in all the concentrations showed significant difference between untreated control and NTB₂ ($P < 0.001$), whilst glucantime only was significantly different with control at high concentrations (200 µg/ml). The difference between glucantime and NTB₂ in all concentrations was significant ($P < 0.001$). In IL-10 expression, a significant difference was observed in all concentrations between untreated control and NTB₂ and G-treated organism ($P < 0.001$). Significant levels in metacaspase expression between these groups were also observed ($P < 0.001$).

DISCUSSION

Although CL is not a deadly disease it results in significant scars and facial disfigurements, thus being clinically important. In recent years, emergence of parasite resistance against the existing drugs has led to the introduction of newer chemotherapeutic agents and vesicular drug delivery systems such as niosomes [15]. In our another study, a clinical trial with the combination of tioxolone along with benzoxonium chloride for improvement of ACL carried out and showed that efficacy of this combination against *L. tropica* has been performed [6]. In present study, we performed several in vitro interactions of tioxolone along with benzoxonium chloride and its niosomal forms and their antileishmanial activities.

In our study, the niosomal forms of this compound were more effective than the simple form against *L. tropica*. The drug entrapment efficiency of niosomal forms was high and in vitro drugs release was gradual; however, benzoxonium chloride was entirely released within 90 min, while tioxolone was released within 240 min. The stability of the niosomal formulation of NTB₂ was greater than NTB₁ formulation and NTB₂ formulation showed no significant change in the particle size after 6 months.

The combination of 2 drugs (tioxolone along with benzoxonium chloride) at higher concentrations, significantly showed a more pronounced leishmanicidal effect on the promastigote

of *L. tropica* in comparison with single forms (T). Also, the inhibitory rate of promastigotes treated with tioxolone along with benzoxonium chloride compared with the gold standard drug (glucantime) was found to be significantly higher. This rate of promastigotes treated with various concentrations of the combination of niosomal forms showed that all formulations significantly exhibited high inhibitory effect in comparison with glucantime ($P < 0.001$). In fact, based on our findings, the simple and niosomal combination of these 2 drugs against promastigote of *L. tropica* were more effective than the standard drug. In the amastigote assays, in tioxolone along with benzoxonium chloride, at the highest concentration of each drug, the mean number of amastigote in each macrophage was 19.33 ± 0.32 , while this number was lower in all of the niosomal formulations at the highest concentration.

In our study, in addition to the good anti-leishmanial effects of the niosomal forms of tioxolone along with benzoxonium chloride, these niosomes are significantly less toxic than glucantime. Also because NTB₂ formulation demonstrated more physical stability, this formulation was preferably selected in this study.

Niosomal formulation of drugs have been demonstrated to be promising controlled drug delivery systems and niosomes also offer successful drug localization in skin with lower side effects, high stability, higher efficacy and cost-effective. Hence, many topical drugs may be developed using niosomal systems [16,17]. It has also been proven in our study that encapsulated drug in niosome reduces drug toxicity.

Apoptosis, a form of programmed cell death, has been described as genetically regulated physiological process of cell suicide. This phenomenon plays an important role in the immunopathogenesis of several parasitic diseases [18]. Apoptosis has already been reported in various unicellular organisms, including *Leishmania*, that this event has been reported in response to a wide range of stimuli, notably leishmanicidal drugs such as miltefosine [19], curcumin [20], pentamidine [21] and the molecule H₂O₂ [22].

Annexin V is a 35-36 kDa Ca²⁺ dependent phospholipid-binding protein, and binds to exposed apoptotic cell surface phospholipid. In *Leishmania* cells which lack any detectable levels of phosphatidylserine, a phospholipid that is exposed at the surface of metazoan cells in response to apoptotic stimuli, and Annexin V binds other phospholipid classes such as phosphatidic acid, phosphatidylethanolamine; phosphatidylglycerol and phosphatidylinositol in *Leishmania* [23,24]. The trans-

location of each of these phospholipids to the outer layer of the plasma membrane, one of the most characteristic features of apoptotic cell death, was observed in dead promastigotes. Our findings showed that on NTB₂ treatment *Leishmania* promastigotes were able to bind to annexin V and induced a type of death that shared most of the features associated with higher number of early apoptotic event (89.13% in 200 µg/ml), as the main mode of action [25].

Given the critical role of immune mediators in the pathogenesis and clinical outcome of CL, it is plausible that functional polymorphisms in immune-related genes contribute to define the outcome of human infection [26]. IL-12 plays a crucial role in both natural and adaptive immunity of host against intracellular pathogens [27]. In our study, with an increase in the concentration of NTB₂, the level of gene expression IL-12 increased significantly, that leads to increase the Th1 cytokine profile, which could make it potentially applicable for treatment of ACL. The expression of IL-12 at all 3 concentrations of NTB₂ was significantly higher than G; therefore, this drug is more effective than the first-line treatment of leishmaniasis (glucantime) in activating the immune system towards recovery, which represent an immunomodulatory mechanism against the leishmanial stages.

High levels of IL-10 production have been associated with active and chronic CL [22], because IL-10 in leishmaniasis has potent immunosuppressive effect on macrophage activation and has strong correlation with disease progression [28]. Our results showed significant reduction in IL-10 gene expression after treatment with NTB₂, which is indicative of Th1 immune response enhancement and parasite death.

Metacaspases (MCAs) are caspase family cysteine peptidases that have been implicated in cell death processes in plants, fungi, and protozoa [29]. The MCA of *L. major* and *L. donovani* has been described as having a function in programming cellular death [29-31]. In this study, we showed that by increasing the concentration of the NTB₂ drug in promastigotes of *L. tropica*, the expression of the MCA gene increases and lead immune responses towards host-protective Th1 response and contributes to apoptosis [32].

These findings indicated that the niosomal form of tioxolone along with benzoxonium chloride with less toxicity to glucantime demonstrated strong anti-leishmanial effects and was able to switch cell-mediated immunity towards recovery, could be promising for the treatment of ACL. The results also represented that tioxolone exhibited a potent lethal activity

alone and more potent in combination. This synergistic effect induced the apoptotic pathways and also up regulation of an immunomodulatory role against as the main mode of action. Therefore, after successful in vitro evaluation of this niosomal formulation, complete international in vivo and clinical protocols should be carried out to introduce an effective topical niosomal drug for the treatment of ACL.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

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