

A Novel Niosomal Combination of Selenium Coupled with Glucantime against *Leishmania tropica*

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Abstract: There is no effective treatment modality available against different forms of leishmaniasis. Therefore, the aim of this study was to improve the penetration and efficacy of selenium and glucantime coupled with niosomes and compared them with their simple forms alone on in vitro susceptibility assays. In this study, the niosomal formulations of selenium and in combination with glucantime were prepared. The size and morphology of the niosomal formulations were characterized and the effectivity of the new formulation was also evaluated using in vitro MTT assay, intra-macrophage model, and gene expression profile. From the results obtained, no cytotoxicity effect was observed for niosomal and simple forms of drugs, as alone or in combination. Niosomal formulations of the drugs significantly showed more inhibitory effects ($P \leq 0.001$) than the simple drugs when the selectivity index was considered. The gene expression levels of Interleukin (IL-10) significantly decreased, while the level of IL-12 and metacaspase significantly increased ($P \leq 0.001$). The results of the present study showed that selenium plus glucantime niosome possess a potent anti-leishmanial effect and enhanced their lethal activity as evidenced by the in vitro experiments.

Key words: *Leishmania tropica*, selenium, glucantime, niosomes, anti-leishmanial effect, cytokine

INTRODUCTION

Leishmaniasis is a neglected disease distributed in more than 98 countries in the world and it has been reported that almost 0.7-1.2 million cases of cutaneous leishmaniasis (CL) occur each year [1,2]. Two common forms of CL, such as anthroponotic cutaneous leishmaniasis (ACL) caused by *Leishmania tropica* (*L. tropica*) and zoonotic CL (ZCL) caused by *L. major*, are present in Iran [3-6].

The clinical manifestations of leishmaniasis are of various form of a self-limiting lesion to damaging mucocutaneous lesions, which depend on the *Leishmanias* species and the type of immune response to the disease [7]. Antimonial drugs (SbV) are considered as the first line of drugs for all forms of *Leishmania* treatment [8]. However, their efficacy is decreased

depending on the *Leishmanias* species and geographical regions [9].

Additionally, all current treatments have limitations derived from their high cost, route of administration, drug resistance, long duration of treatment and especially, serious side effects such as nephrotoxicity, hypokalemia, hepatic and pancreatic toxicity, hypotension and dysglycemia among others. Therefore, there is an urgent need for the development of innovative treatment modalities against leishmaniasis that are safe, inexpensive and easily available to the patients. Furthermore, the discovery of new lead compounds for this disease is a pressing concern for global health programs.

Selenium (Se) is an essential element for human health including antiviral activities, cancer prevention, antioxidant and anti-inflammatory effects [10]. Selenium ions ameliorate the immune response of hosts against various species of viral antigen and bacteria [11]. Several studies have demonstrated appropriate efficacy of selenium nanoparticles against *L. major*, *L. tropica*, and *L. infantum* [12-14].

Vesicular drug delivery is one of the approaches that encapsulate drug. Examples include niosomes, transferosomes, lipo-

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somes, provesicles and pharmacosomes. The benefits of niosomes and liposomes over other conventional forms are their action as a drug reservoir owing to their particulate nature. Niosomes have more stable structures than liposomes even in the emulsified form [15].

Niosomes are drug carriers with a bilayer structure in an aqueous phase which are formed by nonionic surfactants and cholesterol. They exhibit high stability, long shelf life and facilitate the delivery of drug at target site in a sustained mode. In addition, as a novel carrier of drugs, they are biocompatible, biodegradable and nonimmunogenic [16].

In this study, the niosomal formulation of selenium and in combination with glucantime were prepared and their activity was assessed using in vitro MTT assay, intra-macrophage model, and gene expression profile. The size and morphology of niosomal formulation were characterized. The aim of this study was to improve the penetration and effectivity of selenium and glucantime coupled with niosomes and compared with these drugs alone by in vitro susceptibility assays.

MATERIALS AND METHODS

Preparation of drug

Glucantime (Sanofi-Aventis, Paris, France) and selenium dioxide 99.9% (SIGMA-ALDRICH/Lot 079K368021) were purchased from commercial sources in Iran. Glucantime and selenium were diluted in Roswell Park Memorial Institute medium (RPMI-1640) (Biosera, Nuaille, France) to prepare serial dilutions (12.5, 25, 50, 100, and 200 µg/ml) [17] freshly before performing each test.

For combination of glucantime and selenium, first and foremost, the concentrations of selenium remained constant, while glucantime was used in increasing order of concentrations (50, 100, and 200 µg/ml). Secondly, the concentrations of Glucantime were constant and selenium was decreasing in order of concentrations.

In the niosomal combination of glucantime and selenium, at first, the concentrations were based on selenium (selenium plus glucantime niosome) and thereafter, the serial dilutions were based on glucantime (glucantime plus selenium niosome).

Preparation of niosome

Selenium niosome and selenium plus glucantime niosome were prepared using film hydration method [18]. In a round bottom flask, Span 40 (5 g/50 ml), Tween 40 (2.5 g/50 ml)

and cholesterol (2.5 g/50 ml) were dissolved in chloroform. The chloroform was evaporated in a rotary evaporator at 60°C and 4 g for 30 min. The thin layer of film was left to cover the inner walls of the flask. The hydration of obtained film was performed by 5 ml deionized water, in which selenium (1%) and also combination of selenium (1%) and glucantime (1.5%) were dissolved at 55°C for 1 hr. The obtained niosomes were solicated for 30 min by bath sonication. To complete hydration, niosomal formulation of each drug were kept at room temperature (24 ± 1°C) for 24 hr.

Characterization of niosomes

The size dispersion of the niosomes was determined using laser light diffraction method by Malvern apparatus (Malvern Mastersizer X, Malvern, UK). The morphology of niosomal formulations was surveyed using camera that was attached to a light microscope (Zeiss, Oberkochen, Germany), equipped with a computer controlled image analysis system.

Leishmania parasite culture

L. tropica standard strain MHOM/IR/75/Mash2 was cultured in Novy-MacNeal-Nicolle (NNN) medium, incubated at 24°C ± 1 and subcultured in RPMI-1640 (Biosera) supplemented with 15% heat inactivated (at 56°C for 30 min) fetal bovine serum (FBS, Gibco, Carlsbad, California, USA), 1% penesterep (Life Technology, Carlsbad, California, USA).

Murine macrophage cell line culture

J774A.1 ATCC® TIB-67™ was purchased from the Pasteur Institute of Iran (Tehran, Iran) and cultured in DMEM medium supplemented with 10% inactivated FBS, 0.5% penicillin (Sigma, Poole, UK), 0.5% streptomycin (Sigma) and incubated at 37°C with 5% CO₂.

Cytotoxicity of drugs on murine macrophage cells

Prior to the tests on *L. tropica* extracellular promastigote and intracellular amastigote, the cytotoxicity concentrations of glucantime, selenium niosome, selenium plus glucantime, selenium plus glucantime niosome and glucantime plus selenium niosome were determined on J774 cells.

Murine macrophage cells (5 × 10⁴) were cultured in 96 well-microplate and incubated at 37°C with 5% CO₂ for 2 hr. After the incubation period, different concentrations of each drug (12.5-200 µg/ml) were added to each well of the 96-well-microplate and incubated for 72 hr. Thereafter, 10 µl MTT (3-[4,

Table 1. Primers which were used for real-time PCR

Primers	Gene	Forward Sequence (5'-3')	Reverse Sequence (5'-3')	Product size (bp)
Macrophages murine cells	IL-12 P40	CTGGAGCACTCCCCATTCTA	GCAGACATTCCCGCCTTTG	160
	IL-10	CTTACTGACTGGCATGAGGATCA	GCAGCTCTAGGAGCATGTGC	101
	GAPDH	AGCTTCGGCACATATTTTCATCTG	CGTTCACTCCCATGACAAAACA	89
Promastigotes of <i>L. tropica</i>	Metacaspase	CAGCAACAATTCTGGCGATA	AAGTTTGAAGTAAAAGGAGACAATTTGG	140
	RPS18 Ribosomal protein (S18)	GTTGAGGTGCGTGGTCTGTC	TGCAGGTTGCTCAGGAGCTT	166

5-Dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide; Thiazolyl blue) (Sigma Aldrich, St. Louis, Missouri, USA) (5 mg/ml) was added to each well of the microplate and incubated for 3 hr. The wells with medium and parasite without any drugs were considered as untreated control. Thereafter, 100 µl Dimethyl sulfoxide (DMSO) (Merck, Darmstadt, Germany) were added for dissolving formazan crystals and incubated at room temperature in the dark for 1 hr. Optical density (OD) was read at 490 nm using ELISA-reader (Bio Tek-ELX800, Winooski, Vermont, USA). The 50% cytotoxicity concentration (CC₅₀) was determined by probit test in SPSS software version 20 (SPSS Inc., Chicago, Illinois, USA).

Extra-cellular promastigote assay

Promastigotes (1×10^5) were cultured at log phase in 96-well microliter plate and 10 µl of different concentrations of all drugs with 60 µl RPMI-1640 were added to each well and incubated at $24^\circ\text{C} \pm 1$ for 72 hr. Promastigote in medium without any drug was considered as the untreated control, while medium with no promastigote was used as blank. All experiments were performed 3 times. Ten µl of MTT solution in RPMI-1640 (no phenol red) was added to each well and incubated at $24 \pm 1^\circ\text{C}$ for 4 hr. The reaction was terminated by DMSO and read using an ELISA reader at 490 nm. The 50% inhibitory concentration (IC₅₀) was assessed by probit test using SPSS software.

Intra-cellular amastigote assay

Cells (1×10^5 cells) of the J774 murine macrophage cell line were cultured on the slides in sterile plates and incubated at 37°C , with 5% CO₂ for 2 hr. The promastigotes (1×10^6) in the stationary phase were added to each slide and then incubated for 24 hr. After the incubation period, different concentrations of all drugs were added to the slides and incubated at 37°C for 72 hr. At the end, the slides were dried, fixed with methanol, and stained by Giemsa for evaluation under a light microscope. The effectiveness of each drug was evaluated by the mean number of amastigotes in 100 macrophages. Every ex-

periment was performed in triplicate.

Quantitative real-time PCR (qPCR)

RNeasy® mini kit (Qiagen, Chatsworth, California, USA) was used for the extraction of RNA according to the manufacturer's protocol. Nanodrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, Delaware, USA) was used for the determination of quantity and purity of each sample. Then, cDNA was synthesized using the TaKaRa Prime Script™ RT reagent kits (Takara Clontech, California, USA). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) [19,20] was used as reference gene for gene expression of IL-12 and IL-10 in murine macrophage cells (J-774) and RPS18 Ribosomal protein (S18) [21,22] for gene expression of metacaspase in *Leishmania* (Table 1). Quantitative RT-PCR of target cDNA was run on a Rotorgene 3000 cyclor system (Corbett Research, Sydney, Australia). The PCR reaction for mRNA detection was carried out in reaction volumes (10 µl) including 5 µl 2X SYBR Green Supermix (SYBR® Premix Ex Taq™, Takara Clontech), 250 nmol forward and 250 nmol reverse primer, and 1 µl cDNA diluted in RNase-free water. PCR reactions were performed in duplicates and results were normalized to the levels of GAPDH and S18 genes as reference genes. To calculate the relative value of the expression level, $2^{-\Delta\Delta Ct}$ method was used as a relative quantification approach for qPCR data analysis.

Statistical analyses

Data analysis was done using SPSS software version 20. ANOVA and independent *t*-test were used to determine the significant relationship between the treatment groups, also IC₅₀ and CC₅₀ were calculated by probit test. The mean $2^{-\Delta Ct}$ for treatment and the mean $2^{-\Delta Ct}$ for control for each cytokine were compared using GRAPHPAD PRISM 6 (GraphPad Software Inc., San Diego, California, USA). $P < 0.05$ was considered as significant.

The Minimum Standards of Reporting Checklist contains details of the experimental design, and statistics, and resources used in this study.

RESULTS

Niosome preparation

In the first step, 3 different formulations for each drug were prepared and then the determination of the morphology and size was carried out. Selection of the best formulation procedure due to medium spherical multi-layer vesicles (Fig. 1) with sizes of 9.75 ± 0.35 and 5.4 ± 0.14 μm for selenium niosome and selenium plus glucantime niosome was accomplished, respectively.

Cytotoxicity analysis

In the investigation of the cytotoxicity of drugs, various concentrations of these drugs and niosomal formulation of them (12.5-200 $\mu\text{g/ml}$) were applied on macrophage cell-line as harboring cells. The CC_{50} of each drug based on OD was assessed (Table 2).

Based on the cytotoxicity analysis, no toxic effect was observed in all drugs at concentrations of 12.5, 25, 50, 100, and

200 $\mu\text{g/ml}$ and as such, the drugs were used in next steps.

Leishmanicidal effects on extra-cellular promastigotes

The inhibitory activity demonstrated a dose-dependent pattern (Figs. 2, 3). All drugs inhibited the growth of promastigotes effectively; however, selenium plus glucantime niosome showed a higher inhibitory effect (Fig. 2). The IC_{50} values of selenium and selenium niosome were 78.07 ± 5 and 48.2 ± 6.2 $\mu\text{g/ml}$, respectively. Also, the IC_{50} values of selenium plus glucantime niosome and glucantime plus selenium niosome were 16.11 ± 0.99 and 42.17 ± 2.47 $\mu\text{g/ml}$, respectively. All the drugs showed a good inhibition index against the growth rate of promastigotes, but selenium plus glucantime niosome formulation was the best ($P < 0.05$).

Leishmanicidal effects on intra-cellular amastigotes

The mean number of intracellular amastigotes in each macrophage, which were treated with drugs and niosomal formulation, was evaluated. Selenium and selenium niosome signifi-

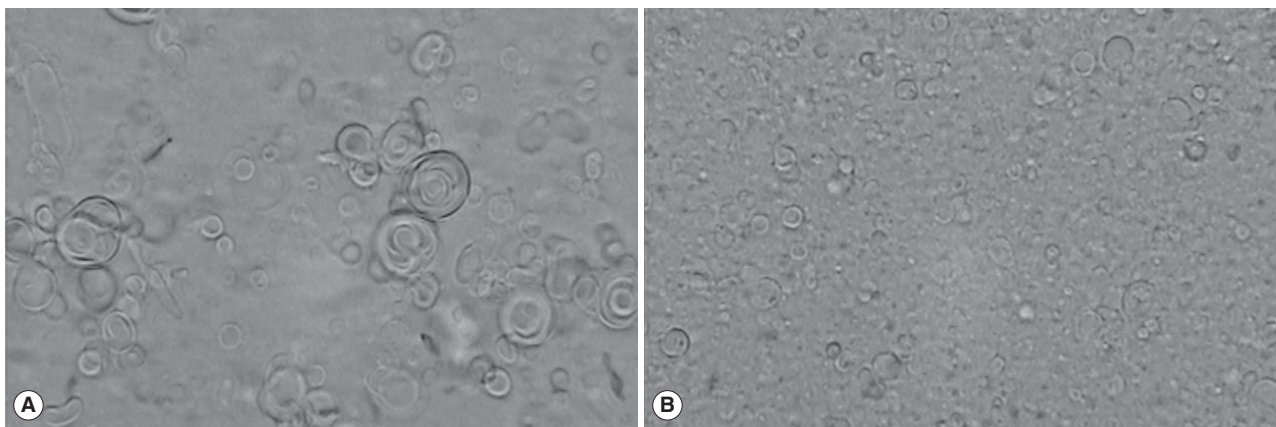


Fig. 1. Microscopic images of Span/Tween 40 (molar ratio = 5:5) selenium niosome (A), and Span/Tween 40 (molar ratio = 5:5) selenium plus glucantime niosome (B).

Table 2. Comparison of the IC_{50} values of selenium, selenium niosome, glucantime, glucantime plus selenium niosome and selenium plus glucantime niosome on *Leishmania tropica* promastigotes and amastigotes, CC_{50} values of drugs on macrophage and SI index

Drug	Amastigote		Promastigote		Macrophage	SI
	$\text{IC}_{50} \pm \text{SD}$ ($\mu\text{g/ml}$)	<i>P</i> -value	$\text{IC}_{50} \pm \text{SD}$ ($\mu\text{g/ml}$)	<i>P</i> -value	CC_{50} ($\mu\text{g/ml}$)	(Selectivity Index)
Glucantime	222.31 ± 28.04	≤ 0.001	144.5 ± 97.3	≤ 0.001	1634	7.35
Selenium	216.18 ± 2.82	≤ 0.001	78.07 ± 5	≤ 0.001	260.51	1.2
Selenium niosome	78.45 ± 1.3	≤ 0.001	48.2 ± 6.2	≤ 0.001	1202	15.32
Selenium plus glucantime niosome	8.67 ± 0.1	≤ 0.001	16.11 ± 0.99	≤ 0.001	1105	127.45
Glucantime plus selenium niosome	14.47 ± 2.22	≤ 0.001	42.17 ± 2.47	≤ 0.001	1511	104.42

IC_{50} , Concentration of drug that caused 50% of growth inhibition of promastigotes and amastigotes; CC_{50} , Concentration of drug that caused 50% of cytotoxicity on macrophages; SI (Selectivity index), the ratio between CC_{50} on J774 cells and IC_{50} against *L. tropica* amastigotes ($\text{SI} = \text{CC}_{50} / \text{IC}_{50} \geq 10$ non-toxic).

cantly decreased the mean number of amastigotes in each macrophage as compared with the glucantime or untreated control (Table 3). Various concentrations of combined niosomal formulation were able to significantly inhibit the number of intracellular amastigotes in macrophages as compared

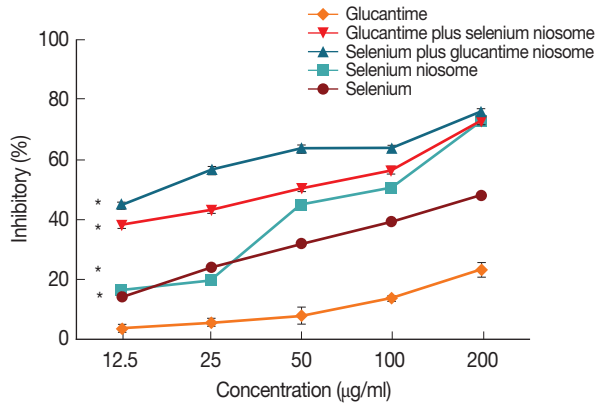


Fig. 2. Comparison of inhibitory effect selenium, selenium niosome, selenium plus glucantime niosome and glucantime plus selenium niosome, on *Leishmania tropica* promastigotes with glucantime as a standard drug, by MTT assay (* $P < 0.05$).

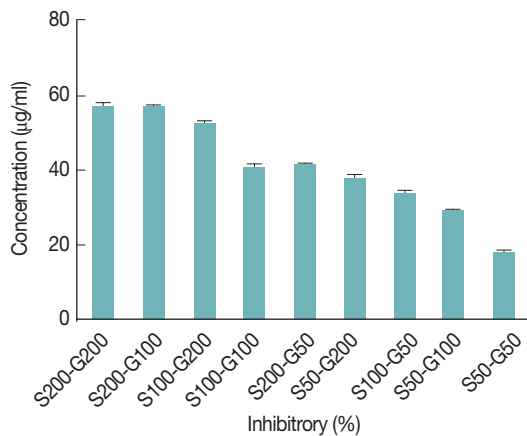


Fig. 3. Inhibitory effect of selenium plus glucantime on promastigotes of *Leishmania tropica*.

with the untreated control ($P < 0.001$) (Table 3).

As regard the effect of all drugs, selenium plus glucantime niosome was significantly more effective than selenium in simple or in combination with Glucantime (Table 4) and the IC_{50} of this formulation against amastigotes was $8.67 \mu\text{g/ml}$ ($P < 0.001$) (Table 2).

Real time

The results of this study showed that the peak of IL-10, as a measure of Th-2, was significantly decreased ($P < 0.001$), although the levels of IL-12 and metacaspase were increased ($P < 0.001$) from concentration of 12.5 to 200 $\mu\text{g/ml}$ (Fig. 4).

DISCUSSION

Antimonials are used as the first line of CL treatment due to their moderate side effects and painful injection [23]. So far, no effective topical drugs have been found to be useful in the treatment of CL, because intracellular parasites live in the macrophage in the deep layer of derm and as such, it is difficult to

Table 4. Comparison of the overall mean effect of various concentrations of selenium plus glucantime on the mean number of amastigotes in each macrophage

Concentrations ($\mu\text{g/ml}$)	Glucantime plus selenium	
	Mean \pm SD	P-value
0 (Untreated control)	36 \pm 0.38	NR
50+50	20.4 \pm 0.4	≤ 0.001
50+100	19.76 \pm 0.45	≤ 0.001
50+200	18.01 \pm 0.4	≤ 0.001
100+50	19.7 \pm 0.3	≤ 0.001
100+100	17.2 \pm 0.37	≤ 0.001
100+200	13.7 \pm 0.46	≤ 0.001
200+50	17.07 \pm 0.31	≤ 0.001
200+100	16.2 \pm 0.41	≤ 0.001
200+200	13.67 \pm 0.5	≤ 0.001

Table 3. Comparison of the overall mean effect of various concentrations of selenium, selenium niosome and, glucantime, selenium plus glucantime niosome, and glucantime plus selenium niosome on the mean number of amastigotes in macrophage

Concentration ($\mu\text{g/ml}$)	Glucantime		Selenium		Selenium niosome		Selenium plus glucantime niosome		Glucantime plus selenium niosome	
	Mean \pm SD	P-value	Mean \pm SD	P-value	Mean \pm SD	P-value	Mean \pm SD	P-value	Mean \pm SD	P-value
0 (Untreated control)	22 \pm 1	NR	22 \pm 0.1	NR	22 \pm 1	NR	32 \pm 0.56	NR	32 \pm 0.56	NR
12.5	21 \pm 0.26	0.51	14.7 \pm 0.1	≤ 0.001	15 \pm 0.2	≤ 0.001	13.95 \pm 0.13	≤ 0.001	15.9 \pm 0.38	≤ 0.001
25	20 \pm 0.75	0.51	13.03 \pm 0.12	≤ 0.001	13 \pm 0.7	≤ 0.001	13 \pm 0.1	≤ 0.001	13.84 \pm 0.63	≤ 0.001
50	15 \pm 0.17	≤ 0.001	12.37 \pm 0.21	≤ 0.001	10 \pm 0.2	≤ 0.001	12.95 \pm 0.05	≤ 0.001	12.85 \pm 0.27	≤ 0.001
100	12 \pm 0.26	≤ 0.001	11.56 \pm 0.52	≤ 0.001	9 \pm 0.05	≤ 0.001	11.06 \pm 0.19	≤ 0.001	10.83 \pm 0.35	≤ 0.001
200	10 \pm 0.62	≤ 0.001	8.03 \pm 0.14	≤ 0.001	8 \pm 0.7	≤ 0.001	6.32 \pm 0.25	≤ 0.001	6.45 \pm 0.14	≤ 0.001

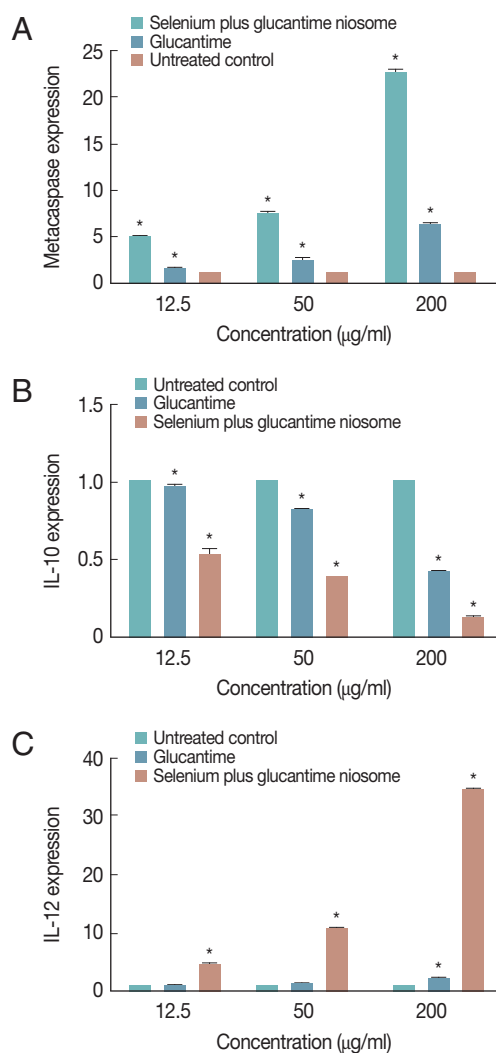


Fig. 4. The gene expression profiles of (A) metacaspase, (B) IL-10, and (C) IL-12p40 on the *Leishmania tropica* treated by the selenium plus glucantime niosome and glucantime in comparison with untreated control (* $P < 0.001$) as measured by using real-time PCR.

access the amastigotes [24].

Therefore, the use of novel delivering of drugs, which is controlled and targeted, is considered to be an effective approach. The application of niosomes and liposomes acting as drug delivery, would be loaded with different drugs. These delivering can control the release drug to the target sites and protect drug from degradation [25]. Niosomes, as compared with liposomes, are more stable chemically and have long half-life during storage [18].

However, small amounts of selenium are required for parasites such as *Leishmania* and *Trypanosoma* [26] but high concentration of this could be toxic and can cause biochemical

and morphological changes such as DNA fragmentation in promastigotes form of *Leishmania*. One of the most important changes that occur in programmed cell death is DNA fragmentation [27].

In this study, selenium niosome, selenium plus glucantime niosome or glucantime plus selenium showed lower cytotoxicity as compared with selenium alone. This result is in agreement with other previous studies which reported that Se NPs demonstrated low toxicity [28,29]. This result also showed that niosomal formulation had the least cytotoxicity because of its targeting delivery of drugs. Hence, we could evaluate the effects of these formulations on the 2 forms of *L. tropica* using in vitro methods.

Several studies reported that Se NPs had significant effects on *Leishmania* species [12,13,30,31]. The results of the present study also showed the anti-leishmanial effects of niosomal formulation of selenium along with glucantime. The IC_{50} values indicated that selenium coupled with glucantime niosome were the most effective formulation against both extra- and intracellular stages of *L. tropica* with the highest safety index (SI=127) and inhibitory level. As regard the evaluation of SI, niosomal formulation was significantly more active against 2 forms of *L. tropica* rather than glucantime and selenium as simple forms.

Combination therapy is the best strategy to prevent drug resistance and also enhance efficacy rate against CL. Other advantages of combination therapy are reduction in the length of treatment, in the administration of doses, side effects and cost [32]. In this study, the niosomal combination of selenium plus glucantime was the most effective on *L. tropica*. Also, other studies have confirmed that combination therapies with meglumine antiminate along with different drugs, such as terbinafine, imiquimod, allopurinol or verapamil could potentiate synergistic effects in the treatment of CL [33-35].

Th1 (Interferon- γ , tumor necrosis factor- α , IL-12, and nitric oxide) and Th2 (IL-4, IL-10, IL-13, and transforming growth factor- β) are 2 different immune response in *Leishmania* infection [36,37]. In the present study, selenium plus glucantime niosome showed an immunomodulatory role for the inhibition of IL-10 and induction of IL-12, which is indicative of an immunomodulatory role of this formulation in cell death as another possible mechanism of action exerted by this combination. Previous studies have shown that the progression of diseases is correlated with IL-10 which played a potent immunosuppressive activity in leishmaniasis, such as suppression of

macrophage activation [38]; the biological processes leading to cell death are multifactorial. Cell death process in protozoa, plants, and fungi is due to the expression of metacaspase as cysteine peptidases [39].

The metacaspase in *L. major* and *L. donovani* plays an important role in the programmed cell death pathways [39-42].

The results of the present study showed that metacaspase was increased in promastigote form of *L. tropica* treated with selenium plus glucantime niosome. Also, increasing the expression level of metacaspase gene contributed to apoptosis in this parasite, and these findings obtained during the study are in line with the results of previous study [41].

The results of the present study showed that selenium niosome possesses a powerful anti-leishmanial effect and enhanced potent lethal activity especially in combination with glucantime in niosomal form as evidenced by the in vitro experiments. Selenium plus glucantime niosome inhibited Th-2 cytokine, induced the Th-1 cytokine mediators and stimulated an immunomodulatory role against the different stages of *L. tropica*. Such progression of lethal action in in vitro model would be prerequisite for performing further investigation in clinical settings.

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CONFLICT OF INTEREST

The authors have no conflicts of interest.

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