

Original Article

Anti-Leishmanial Effects of a Novel Biocompatible Non-Invasive Nanofibers Containing Royal Jelly and Propolis against Iranian Strain of *Leishmania major* (MRHO/IR/75/ER): an In-Vitro Study

Mohsen Mahmoudi¹, Bitra Mehravi², Mohammad Shabani³, Ramtin Hadighi¹, Alireza Badirzadeh¹, Ahmad Dehdast³, Ghazale Chizari-Fard³, Vahid Pirhajati-Mahabadi⁴, Sekineh Akbari⁵, *Fatemeh Tabatabaie¹, *Mehdi Mohebali⁶

¹Department of Parasitology and Mycology, School of Medicine, Iran University of Medical Sciences, Tehran, Iran

²Department of Medical Nanotechnology, School of Medicine, Iran University of Medical Sciences, Tehran, Iran

³Department of Biochemistry, School of Medicine, Iran University of Medical Sciences, Tehran, Iran

⁴Neuroscience Research Center, School of Medicine, Iran University of Medical Sciences, Tehran, Iran

⁵Department of Parasitology and Mycology, School of Medicine, Isfahan University of Medical Sciences, Isfahan, Iran

⁶Department of Parasitology and Mycology, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran

*Corresponding authors: Dr Mehdi Mohebali, E-mail: mohebali@tums.ac.ir, Dr Fatemeh Tabatabaie, E-mail: tabatabaei.f@iums.ac.ir

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Abstract

Background: Current medications especially the pentavalent antimonial compounds have been used as the first line treatment of cutaneous leishmaniasis (CL), but they have limitations due to serious side effects such as drug resistance, cardio and nephrotoxicity, and high costs. Hence, the demand to find more usable drugs is evident. Synthesis and development of natural, effective, biocompatible, and harmless compounds against *Leishmania major* is the principal priority of this study.

Methods: By electrospinning method, a new type of nanofiber were synthesized from royal jelly and propolis with different ratios. Nanofibers were characterized by Scanning Electron Microscope (SEM), Transmission Electron Microscopy (TEM), Thermogravimetric Analysis (TGA), Contact angle, and Fourier-transform infrared spectroscopy (FTIR). The Half-maximal inhibitory concentration (IC₅₀), Half-maximal effective concentration (EC₅₀) and the 50% cytotoxic concentration (CC₅₀) for different concentrations of nanofibers were determined using quantitative calorimetric methods. Inductively coupled plasma-optical emission spectrometry (ICP-OES) and flow cytometry were performed as complementary tests.

Results: The results showed that the proposed formulas provide a new achievement that, despite the significant killing activity on *L. major*, has negligible cytotoxicity on the host cells. Royal jelly nanofibers have significantly shown the best 72 hours results (IC₅₀= 35 µg/ml and EC₅₀=16.4 µg/ml) and the least cytotoxicity.

Conclusion: This study presents a great challenge to introduce a new low-cost treatment method for CL, accelerate wound healing, and reduce scarring with minimal side effects and biocompatible materials. Royal jelly and propolis nanofibers significantly inhibit the growth of *L. major in-vitro*.

Keywords: *Leishmania major*; Nanofiber; Propolis; Royal jelly; *In-vitro*

Introduction

The World Health Organization (WHO) has introduced leishmaniasis as one of the nine major

parasitic diseases in tropical and subtropical regions of the world (1). Currently 92 countries

are affected by clinical forms of the disease and the number of new cases of cutaneous leishmaniasis (CL) is estimated at one million annually and 80% of the disease occurs in the Eastern Mediterranean region (2).

At present, due to the lack of a desirable vaccine, pentavalent antimony compounds are still the first line of treatment for leishmaniasis in Iran and other countries in the world. Meglumine antimoniate (Glucantime®) is suggested by the WHO for the treatment of all types of leishmaniasis but is associated with hematologic, hepatic, and renal side effects (3, 4). However, after many years, there is still no definitive treatment for it, and research is ongoing to find new drugs with fewer side effects and more effectiveness. Hence, achieving novel drugs and applications, also new therapeutic strategies are needed. Replacing new tissue through the restoration and regeneration of damaged tissue is one of the treatment strategies for leishmaniasis, which is part of tissue engineering. Nanofibers are a new type of material that recently have enormous applications in medicine as biocompatible and biodegradable compounds that can stimulate the immune system. Currently, the tendency is more toward natural resources such as plants or animal products. Plenty of research has been done on the health benefits and pharmaceutical properties of bee products.

Royal jelly; Chemically, is highly complex, rich in protein, and contains essential amino acids, vitamins, probiotics, fiber, Omega 3, sterols, and phosphorus compounds such as acetylcholine (5). Royal Jelly Proteins (RJPs) are reported to be hydrolyzed by protease N, resulting in potent antioxidant activity (6). Antibacterial, anti-tumor, antioxidant, anti-inflammatory, and anti-viral properties have been reported from this valuable substance. 10-hydroxy 12-decenoic acid (a bioactive component), has immune regulating properties (7–9). Royal jelly is also an important source of gelatin (precursor of collagen) and plays a very important role in tissue repair (10, 11).

Propolis; is a greenish-brown substance and source of resinous material produced by bees, and it has protective properties for bee larvae. It is collected by bees from some trees (12). Studies have shown this substance has high potential in eradicating various microorganisms and parasites (13, 14). Propolis has shown remarkable healing activities for wounds, burns, and oral lesions, and accelerates tissue repair by stimulating glycosaminoglycan synthesis (15–18). The biological activity of propolis is due to its phenolic compounds such as flavonoids (19).

According to the National Science Foundation definition, nanomaterials are materials that are less than or equal to 100 nanometers in diameter (One-dimensional nanostructures), and their special properties and wide applications concerning the mass state, they have aroused great interest among researchers (20–22) and have biocompatible and biodegradable contents that are capable of stimulating the immune system (23).

Electrospun nanofibers have attracted the attention of researchers due to their unique characteristics such as morphology (dimensions), large surface-to-volume ratio, and large inter-intra-connected pores (24). Mainly, the fibers are collected in a non-woven layer called a nanoweb. In this process, a high-voltage electric field pulls the fibers from a polymer solution through a small nozzle, which causes the production of fibers with nanometer dimensions (25).

An ideal dressing should be easy to remove from the wound without damaging the tissue, absorb wound secretions, prevent superficial infection, and allow gas exchange between the wound and the environment. They have many applications in medicine (tissue engineering and biosensors) (26) and drug delivery (DDS) as carriers, protection of the drug molecules, and so on. This new technology also makes it possible to use highly toxic drugs (27).

In this study, a unique innovation has been carried out. For the first time, the nanofibers of

natural products, including royal jelly and propolis, have been used as a safe topical remedy for CL. The current study strived to in-vitro evaluation of the propolis and royal jelly containing Glucantime® and without Glucantime® in the form of nanofibers on *L. major*.

Materials and Methods

Solutions

All required solutions are listed in Table 1 along with their percentages. The materials were converted into nanofibers via electrospinning processing (Propolis Hydro- alcoholic extracts, Royal jelly, and Polyvinyl alcohol (PVA) aqueous solution with different proportions). PVA is a stabilizing agent for these compounds (PVA, Specific weight of PVA= 7200 grams/mole, Sigma Aldrich, Saint Louis, MO, USA). PVA can enhance crosslinking in the aqueous media. In this investigation, Glucantime® vials (Rhone Poulenc Rorer, Paris, France) are used to test.

In the magnetic stirrer method with a hot plate, the mixture is prepared by placing the sample on the Stirrer (with a magnet) overnight at a gentle temperature of about 40-centigrade degrees. When precise and controlled temperatures are required, heating magnetic stirrers are particularly useful. The solution was passed directly through microporous filters. Each 5 ml ampoule of Glucantime® contains 1.5 grams of meglumine antimonite equivalent to 0.405 grams of pentavalent antimony.

In the combined nanofibers containing Glucantime®, there are equal amounts of royal jelly and propolis, for example, nanofibers plus 2% Glucantime® contains 19.6% royal jelly and propolis.

Grouping and preparation of nanofibers

The studied groups are as follows:

Group 1: Royal jelly+ PVA

Group 2: Propolis+ PVA

Group 3: Royal jelly+ Propolis+ PVA

Group 4: Royal jelly+ Propolis+ Glucantime® 2%+ PVA

Group 5: Royal jelly+ Propolis+ Glucantime® 4%+PVA

Group 6: Royal jelly+ Propolis+ Glucantim® 6%+ PVA

Control Group (7): Glucantime® (standard drug)

The nano phase of the study

Nanofibers synthesis by electrospinning

The electrospinning technique has been employed to synthesize the examined nanofibers. The present study aimed to optimize the electrospinning parameters to prepare appropriate nanofibers. Feasibility and experimental studies were carried out to estimate and design parameters. To confirm the proper morphology and stability of the nano scaffold, Scanning Electron Microscope (SEM) and Transmission Electron Microscopy (TEM) of all samples' surfaces were taken before testing. The electrospun fibers, confirmed by SEM and TEM, were collected on the aluminum foil surface (under the parameters obtained from the pilot studies) via the electrospinning machine (Lab-Scale Electrospinning Machine-Electroris®, FNM Company, and Iran).

The drug release rate of the combined nanofiber containing Glucantime® by ICP-OES technique.

About 13 mg of propolis/ Royal Jelly/ PVA nanofibers containing Glucantime® (6%) was weighed and placed in a 12 kDa dialysis bag with 5ml PBS at PH= 7.4. The complex was placed in a beaker containing 50 ml of PBS (pH= 7.4) and then on a shaker at 37 °C. Up to 48 h, at different intervals, 2 ml of buffer was removed for analysis and replaced with the same amount of fresh buffer. Induction coupled plasma emission spectrometry (ICP-OES) at 217.5 nm was used to study the drug loading and release rate of Glucantime® at different time intervals in the solution medium. Dialysis bags should be prepared before the tests (28).

Characterization

Morphological analysis

In the present study, nanofibers were suc-

cessfully fabricated by electrospinning. Prior to in-vitro tests, SEM and TEM analysis chemically and morphologically characterized the nanostructures. SEM analyzed the nanofibers' surface morphology, and their size, shape, and distribution were analyzed by TEM with better spatial resolution than SEM.

Fourier transform infrared (FTIR), Thermogravimetric analysis (TGA), and contact angle were utilized to characterize the three selective nanofibrous structures.

Analysis of chemical interactions by FTIR

FTIR was used to characterize the presence of specific chemical groups and the chemical interactions between royal jelly-PVA, propolis-PVA, and combination (royal jelly+ propolis)-PVA nanofibers (Jasco FP-6300 Spectrofluorometer- Japan). The spectra were recorded in the 4000-650 cm^{-1} spectral range during 32 scans with 4 cm^{-1} resolution (29, 30).

Measurements of wettability (Hydrophobicity and Hydrophilicity) of nanofibers by contact angle

The contact angle was measured by the contact angle measurement system model OCA 15 plus-Germany. Contact angle measurements of three electrospun nanofibers were conducted at room temperature (25 °C). If the contact angle between the surface and the drop is greater than 90 degrees, a hydrophobic surface exists. For a super hydrophobic surface, the contact angle should be greater than 150 degrees; in this case, the water drop will be spherical and roll off the surface. If the contact angle between the surface and the water drop is less than 90 degrees, the surface is considered hydrophilic. In this case, the drop of distilled water is absorbed on the surface of the material (31).

Thermogravimetric analysis (TGA)

TGA measurements were performed on royal jelly, propolis, and royal jelly/propolis combination nanofibers (LABSYS evo 1150-SETARAM-FRANCE) under nitrogen atmosphere, in the temperature range 50 to 1000 °C

with different heating rates ranging from 1 °C/min up to 50 °C/min) (32).

Nanofibers cross-linking for stability

Nanofibers were treated in a vacuum-sealed desiccator containing 10 mL 0.25% Glutaraldehyde (GA, Merck, Germany), for 24 h. at room temperature, and then they were dried at the same condition overnight (Cross-linking increases moisture and heat resistance) (33). The crosslinking reaction was confirmed by SEM analysis.

The parasitology phase

In-vitro culture of *Leishmania* in RPMI1640 medium

Leishmania major Iranian strain (MRHO/IR/75/ER) was kindly received from the reference leishmaniasis lab, the School of Public Health, Tehran University of Medical Sciences. *Leishmania major* isolates were transmitted into Novy-McNeal-Nicolle (NNN) medium and then subcultured in RPMI 1640 medium to produce massive volume. The minimum required amount of parasites at the log phase is about 1×10^6 parasites per milliliter which confirm by Trypan Blue (34).

Evaluation of anti-leishmanial activity of nanofibers on *L. major* Promastigotes by MTT test (IC₅₀)

In-vitro drug susceptibility assay was presented as IC₅₀ (50% inhibitory concentration) which was determined using nonlinear regression analysis. MTT (3-[4, 5-Dimethylthiazol-2-yl]-2, 5 diphenyl tetrazolium bromide) assay was used to measure cellular metabolic activity as an indicator of cell viability and cytotoxicity. The Elisa microplates are used to perform the MTT test. 100 μl of different concentrations of nanofibers (25, 50, 100, 200 $\mu\text{g}/\text{ml}$) was added to 10^6 Log phase promastigotes/ 100 ml of culture medium in each well of the 96-well plate. Promastigotes were allowed to grow for 24, 48 and 72 hours at 24 °C, and then their viability was assessed by the MTT quantification test. After mentioned times, 20 μL of te-

trazolium solution was added to each well and incubated at 24 °C for 4 hours. After incubation, the formazan crystals were dissolved by adding 80 µl DMSO (Dimethyl sulfoxide) and the plate was left in the dark for 15 minutes, then the absorbance (Optical Density= OD) was measured at 570 nm using an Elisa microplate reader spectrometer: Cell Viability= $[(AT-AB)/(AC-AB)] \times 100$ (34).

In-vitro assessment of the drug effects on the amastigote form (the half-maximal effective concentration or EC₅₀)

Metacyclic promastigotes were isolated from promastigote cultures in the late stationary phase and were augmented to the murine macrophages' cells line J774 (purchased from the Pasteur Institute Animal Laboratory, Tehran, Iran). In the culture medium, macrophages are highly adherent cells, so they adhere to the bottom of the container and are capable of phagocytosis. Trypan blue exclusion assay was one of the most common methods for cell viability measurement (90 µl Trypan blue 0.2% in saline containing 01% Sodium Azide: 2 minutes (35, 36). Viability is described as the percentage of live cells in a total population (live and dead cells).

EC₅₀ values were assessed using six-well plates and methanol fixed, the Giemsa-stained, and microscopically examined (400× magnification) to count the number of intracellular amastigotes per hundred infected macrophages (36).

In-vitro evaluating the cytotoxicity of nanofibers by MTT assay (The 50% cytotoxic concentration or CC₅₀)

Murine macrophages (cell line J774) were used to evaluate the biocompatibility of nanofibers. IC₅₀ values were evaluated by MTT test using routine laboratory procedures (37).

Selectivity Index

The selectivity index (SI) for each nano-drug can be estimated as the ratio between reported cytotoxic values (CC₅₀) to the reported antileishmanial (promastigotes and amastigotes)

activity value (IC₅₀ and EC₅₀). The higher the SI ratio, theoretically, the drug will be more effective and safer during the in vivo treatment of cutaneous leishmaniasis.

Flow cytometry analysis

Since the principal purpose of this investigation was to synthesize natural-based nanofibers to treat cutaneous leishmaniasis, the efficiency of different concentrations of nanofibers against promastigotes of *L. major* was studied by the flow cytometry method. The apoptotic, necrotic, and viable cell percentages for each specimen were evaluated by analyzing the flow cytometry diagrams (10⁶ promastigotes/ml after 24 h treatment with 200 µg of different nano-drugs) (38).

Statistical analysis

All statistical analyses were conducted with Prism, version 9 (Graph Pad Software, San Diego, CA). The data were analyzed using one-way analysis of variance (ANOVA), and the Tukey test. A P-value less than 0.05 was typically considered statistically significant. All experiments were accomplished in triplicate.

Results

To confirm the form and physicochemical structure of nanofibers, a number of characterization analyzes were performed;

SEM analysis

Surface morphology of the nanofibers analysis by SEM (EVO LS₁₀, ZEISS) with a voltage of 20 KV and flow rate of 0.7 ml/h. As can be seen, smooth electrospun nanofibers formed without any formation of beads (Fig. 1. D and E).

TEM Analysis

The quality, shape, and size of nanofibers analyzed by TEM (LEO 906,100kv, Germany, and ZEISS) is shown in Fig. 2.

FTIR Analysis

The FTIR spectra in the mid-infrared region

in the range of 4000-650 cm^{-1} related to propolis, royal jelly, PVA, and nanofibers are shown in Fig. 3. This spectrum agreed with the results reported by other researchers (39–40). In the propolis spectrum, two sharp peaks are observed at 2919 and 2850 cm^{-1} , which are related to the symmetric and asymmetric C-H stretching vibrations of CH₂ groups in saturated hydrocarbons. Other peaks in the range of 1160-1735 cm^{-1} indicate the presence of flavonoids and lipids in propolis. Some peaks are slightly different from the spectrum reported by others, which is probably related to the difference in the origin of propolis (40).

The spectrum of royal jelly (Fig. 3) had vibration bands in the range of 3406, 1643, 1546, 1410, 1243, 1059, 1053 and 921 cm^{-1} , which are probably related to the present chemical groups in the royal jelly. The broadest and strongest band with a peak at 3406 cm^{-1} was due to the stretching of the O-H groups from water and the N-H stretching vibrations of the amine (41, 42). Another dominant region was between 1546 and 2079 cm^{-1} , and two bands were observed at 1643 and 1546 cm^{-1} . The band with the peak at 1643 cm^{-1} corresponded to amide I, which is probably related to C-O stretching vibrations belonging to the backbone structure of proteins. In addition, the peak observed at 1546 cm^{-1} can be due to N-H bending and C-N stretching vibrations of amide II. The information related to the secondary structure of the protein is placed in the spectral domain of Amide-I and Amide-II (43).

The FTIR spectrum of PVA shown in Fig. 3 indicates that most of the prominent peaks are related to hydroxyl and acetate groups. Also, the prominent bands observed at 3428 cm^{-1} can be related to the O-H stretching of intermolecular and intramolecular hydrogen bonds. In addition, the vibrational band observed at 2911 cm^{-1} indicates the C-H stretching of alkyl groups. The peaks between 1715 cm^{-1} may be related to the C-O stretching of the remaining acetate group of PVA. As can be observed (Fig. 3), compared to raw propolis, the peak of prop-

olis nanofibers at 3326 shows a wider band, which corresponds to the OH group of PVA. The presence of peaks of 2939 cm^{-1} and 1707 cm^{-1} are associated with C-H and C-O acetate groups, respectively. Peaks of 1089 cm^{-1} to 1637 cm^{-1} are related to propolis flavonoids and lipids. Compared to propolis and PVA peaks, these outcomes reveal the formation of Propolis-PVA nanofibers.

Concerning royal jelly-PVA nanofibers, as can be seen, the peak point at 3394 represents the OH group of PVA, which due to its band wideness, covers the N-H groups of royal jelly amines. The band with a peak point at 2938 cm^{-1} corresponded to C-H groups and the peak points at 1655 cm^{-1} and 1549 cm^{-1} are associated with amide-type1 and amine-type 2 of royal jelly, respectively. Compared to the peaks of Royal jelly and PVA, these results confirm the formation of Royal jelly - PVA nanofibers.

Regarding the spectrum of propolis-royal jelly nanofiber (Fig. 3), the widening of the band at point 3412 cm^{-1} compared to propolis is due to the presence of the O-H group of PVA. In the IR spectra of royal jelly-PVA nanofibers, one peak was observed at 2940 cm^{-1} referring to the C-H groups, and other peaks in the range of 1088-1688 cm^{-1} revealed the presence of flavonoids and lipids in propolis. The presence of peaks at 1640 cm^{-1} and 1519 cm^{-1} are attributed to amide-type1 and amine-type 2 of royal jelly, respectively. In comparison with propolis, PVA, and royal jelly peaks, the results confirm the formation of propolis-PVA-royal jelly nanofibers.

Contact angle analysis

The contact angle indicates the hydrophilicity of the compounds. Contact angle analysis (the wetting properties) revealed that the contact angles of royal jelly (1), propolis (2), and royal jelly/propolis combination (3) nanofibers were 81, 84 and 79 degrees respectively (Fig. 4). Compared to the three-electrospun samples, the contact angle of the royal jelly/propolis combination nanofibers decreased to 79 degrees. It can

be concluded that the hydrophobicity of the mentioned materials has increased in the combined form.

TGA analysis

TGA investigations on the thermal degradation of royal jelly nanofibers, propolis nanofibers, and a combination of royal jelly and propolis nanofibers in nitrogen are reported. As can be seen in Fig. 5, the sigmoid curve of these nanofibers shows that the amount of weight loss of the combined samples at 350 to 450 degrees Celsius (11.009 mg) is more than the other two samples. The sigmoid curve of propolis and royal jelly overlaps in some parts and is 10.396 mg and 10.398 mg respectively. The weight of combined nanofiber hydrocarbons is higher due to the simultaneous presence of propolis and royal jelly. As a result, the amount of carbon burned and weight loss is greater.

Assessing drug release from the combined Nano medication

Since the combined nanofibers contain antimony metal, ICP-OES (Inductively coupled plasma-optical emission spectrometry) was performed to investigate the drug release rate and Kinetic of the antimony from the nanofibers scaffold at different times. For this purpose, the nano-drug containing Glucantime® 6% was employed. Figure 6 demonstrates the 48 h drug release rate from the nanofibers scaffolds. As can be seen, within 6 h, the drug release rate from scaffolds was 42.30%. In other words, the release rate was accelerated in the early hours (Nearly half of the loaded drug). Within 18 h (from 6 to 24 h), about 20% of the drug was, and within 24 h (from 24 to 48 hours) about 10% of the drug was released from the scaffold. As a result, after 24 h, the drug release rate gradually decreased over time. Up to 48 h, about 64% of the drug was released from the scaffold, approximately 54% of which occurred in the first 24 h (Table 2).

Biocompatibility of the nanofibers

To investigate the biocompatibility of the

tested nanofibers, the proliferated macrophages were incubated (37 °C in 5% CO₂) in the vicinity of the combined nanofibers (royal jelly+ propolis), and after the preparation processes, they were analyzed using the SEM technique. As can be seen, healthy elongated macrophages are surrounded by nanofibers (Fig. 6).

In-vitro inhibitory assessment of Glucantime®-free nanofibers and nanofibers containing different concentrations of Glucantime® against *L. major* promastigotes (IC₅₀)

To assess the Royal jelly, propolis, and combination of Royal jelly-propolis nanofibers (Fig. 7A) activity against *L. major* promastigotes, IC₅₀ (µg/ml) values were evaluated by MTT assay. Promastigotes were incubated and exposed to various concentrations of nanofibers (25, 50, 100 and 200 µg/ml) at 24, 48 and 72 h intervals. As seen in Fig. 7A, IC₅₀ values of royal jelly against promastigotes were 36.7, 26.5 and 22 µg/ml at 24, 48, and 72 h and for propolis, nanofibers were 48, 38.6, 36.5 µg/ml respectively. But in the case of nanofibers in combination, the promastigote inhibitory effect of combined nanofibers during 48 h was about six times higher than its 24 h effect, and the IC₅₀ of the drug dropped from 79 µg/ml to 28 µg/ml. As seen in the Fig. 7B, the nanofibers containing 6% Glucantime® significantly inhibited the growth of promastigotes (IC₅₀= 98 µM/ml) in the first 24 h and they were more effective than the other two nanofibers at this time (P< 0.05). After 72 h, the inhibitory activity of all nanodrugs was enhanced. The 72 h efficacy of Glucantim® 4% was the best compared to other Glucantim® compounds (IC₅₀= 40 µg/ml) (P< 0.05). IC₅₀ of all studied groups at different times are listed in Table 3.

In-vitro cytotoxicity Effect of Glucantime®-free nanofibers and nanofibers containing different concentrations of Glucantime® on *L. major* amastigote (EC₅₀)

As shown in Fig. 8A, the proposed nanofibers had the highest anti-proliferative effect on *L. major* amastigotes after 72 h, that is, their

lethal impact has augmented over time. In an overview, royal jelly nanofibers with EC_{50} of 25.5, 19.6 $\mu\text{g/ml}$, and 16.4 $\mu\text{g/ml}$ at 24, 48 and 72 h respectively, have demonstrated the highest efficacy. EC_{50} for the propolis nanofibers group was 24.9, 20.3 and 17.6 $\mu\text{g/ml}$ respectively at the different times.

Based on the outcomes (Fig. 8B), compared to other Glucantime® compounds, the 72 h efficacy of combined nano-drugs containing 6% Glucantime® had the best cytotoxic result on amastigotes (EC_{50} = 18.8 $\mu\text{g/ml}$). These EC_{50} values for combined nanofibers containing 2%, and 4% Glucantime® were about 23 $\mu\text{g/ml}$ for 72 hours. EC_{50} of all studied groups at different times are listed in Table 4.

Invitro cytotoxicity assessment of Glucantime®-free nanofibers and nanofibers containing different concentrations of Glucantime® on macrophages (CC_{50})

The studied nanofibers showed noticeably low toxicity on macrophages. In other words, in comparison to studied concentrations (25–100 $\mu\text{g/ml}$), their cytotoxic effect (CC_{50}) occurs in extremely high concentrations (2500–3000 $\mu\text{g/ml}$). As seen in Fig. 9A, in the first 24 h, royal jelly indicated the least cytotoxic effect on macrophages (CC_{50} = 3500 $\mu\text{g/ml}$), and nanofibers in combination demonstrated the highest cytotoxicity value (CC_{50} = 2492 $\mu\text{g/ml}$). After 24 h, the cytotoxicity gradually increased in all groups. Compared to the studied nano-drugs, royal jelly had the lowest cytotoxicity at all times. In the first 24 h, the cytotoxicity of the nanofibers in combination was higher than that of propolis nanofibers (CC_{50} : 2500 $\mu\text{g/ml}$ versus 2785 $\mu\text{g/ml}$ respectively), but after 48 h, this ratio was reversed, and the cytotoxicity of propolis exceeded that of the nanofibers in combination (CC_{50} : 2444 $\mu\text{g/ml}$ versus 2025 $\mu\text{g/ml}$). After 72 h, all nano-drugs cytotoxicity was enhanced. In general speaking, the mentioned nano-medication had insignificant cytotoxicity on macrophages, and the drugs are completely safe and can be recommended for medical purposes.

According to Fig. 9B, the greatest cytotoxic effect on macrophages was concerned with the combined nano-fiber plus 6% Glucantime® with a CC_{50} of 2395 $\mu\text{g/ml}$, which dropped to 1863 $\mu\text{g/ml}$ after 72 hours. In the first 24 h, the combined drugs plus 6% were more toxic to macrophages than Glucantime® alone (CC_{50} = 2900 $\mu\text{g/ml}$), but after 72 h, the cytotoxicity of Glucantime® exceeded that of other nano-drugs (CC_{50} = 1720 $\mu\text{g/ml}$). Among the combinations containing Glucantime®, in the first 24 h, the 2% compound revealed the least cytotoxicity on macrophages, but its lethal effect gradually increased from 48 to 72 hours and even exceeded that of the 4% compound. The CC_{50} value of Glucantime®% 2 at 48 h and 72 h was 2046 $\mu\text{g/ml}$, 2010 $\mu\text{g/ml}$, and the CC_{50} value of Glucantime®% 4 at the mentioned times was 2537 $\mu\text{g/ml}$, 2252 $\mu\text{g/ml}$, respectively. CC_{50} of all studied groups at different times are listed in Table 5.

Selectivity Index

The selectivity index was calculated using the ratio of CC_{50} to IC_{50} values (promastigotes) and the ratio of the CC_{50} to EC_{50} values (amastigotes) for all nanofibers in 24, 48 and 72 hours are shown in Table 6.

Flow cytometry analysis

Flow cytometry analysis was performed to assay promastigotes necrosis and apoptosis using 200 $\mu\text{g/ml}$ of our studied drugs in 24 h (Fig. 10). For this purpose, nanofibers with various concentrations of Glucantime® as well as combined royal jelly/propolis nanofibers were employed. As can be seen in the diagram, the combined drugs (Royal jelly/propolis) caused 35.22 % cell apoptosis (LR+UR) and 9.3% cell necrosis within 24 h, and overall induced 44.51 % cell death. These values for the control group (Annexin-p) are 18.62% (15.60%+3.02) apoptosis, 9.8% necrosis, and a total of 29% cell death. Compared to other combined drugs with Glucantime®, the combination of 4% significantly showed the highest lethality percentage and caused cell death of 40.02%, which in-

cluded cell apoptosis of 30.6% (24.64+5.96) and necrosis of 9.42%. For the combination of 2%, cell death induced by necrosis was 12.33%.

The current study involved several challenges, including political issues and international sanctions, difficulties in obtaining laboratory

materials, lack of quick and timely access to the required laboratory equipment, and tight restrictions on financial issues. Moreover, most importantly, the COVID-19 pandemic has significantly influenced laboratory research and practice.

Table 1. Required solutions and concentrations

Required solutions	Solution type	Concentration	Notes
Royal jelly	Aqueous	17% w/v	Prepared with distilled water
Propolis extract	Hydro- alcoholic	10% w/v	Heater Stirrer method(more transparency)
PVA	Aqueous	10% w/v	The stabilizer agent

Table 2. The release rate of antimony from electrospun nano-fiber containing %6 Glucantime® base on time by, ICP-OES (Inductively coupled plasma - optical emission spectrometry) technique

Time (Hour)	0.25	0.5	0.75	1	1.30	2	3	4	6	24	48
Release amounts (%)	3.07	6.92	9.23	13.89	17.69	23.07	28.46	34.61	42.30	53.07	63.84

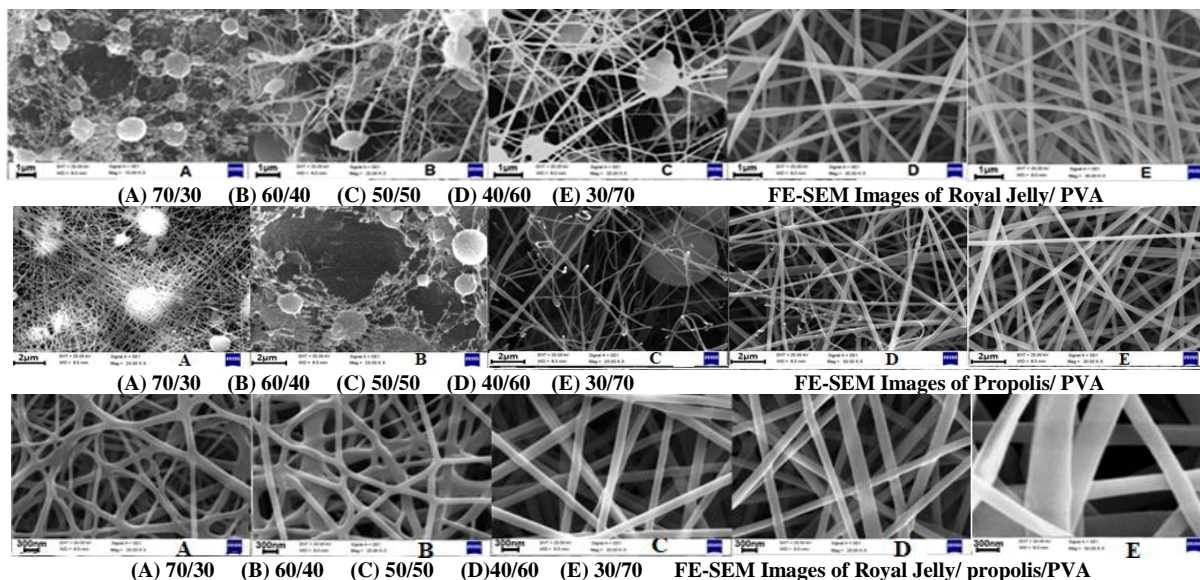


Fig. 1. FESEM images for the studied nanofibers with different proportions of royal jelly, propolis and PVA. (Sample D; /40% Royal jelly and Propolis equally/ 60% PVA; was selected for study)

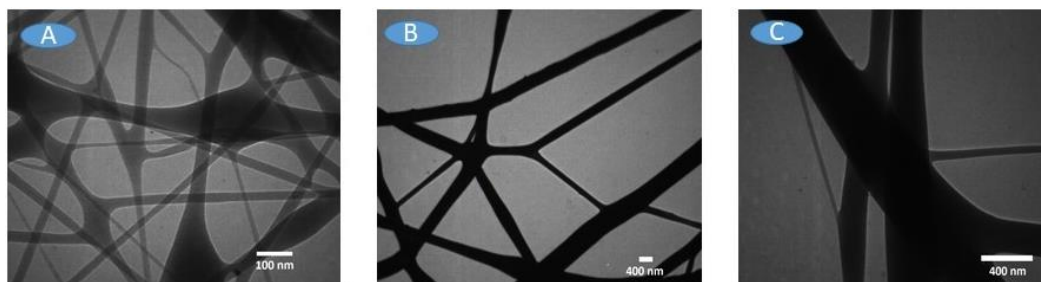


Fig. 2. TEM images for the 3 selective studied nanofibers: (A) Royal jelly+ Propolis, (B) Royal jelly (C) Propolis

Table 3. IC₅₀ of all experimental groups over time

Nano drug\ Time	24 h	48 h	72 h
Royal jelly	36.7	26.5	22
Propolis	48	38.6	36.5
Roy./ Prop.	79	28	23.5
Glucantime® 2%	158	62	59.3
Glucantime® 4%	182	122	40
Glucantime® 6%	98	75	55
Glucantime®	95.3	58	34

Table 4. EC₅₀ of all experimental groups over time

Nano drug\ Time	24 h	48 h	72 h
Royal jelly	25.5	19.6	16.4
Propolis	24.9	20.3	17.6
Roy./ Prop.	31	21	17.5
Glucantime® 2%	67.8	42	23.6
Glucantime® 4%	37.8	29.5	23.3
Glucantime® 6%	30.8	23.8	18.8
Glucantime®	53	36.5	21.3

Table 5. CC₅₀ of all experimental groups over time

Nano drug\ Time	24 h	48 h	72 h
Royal jelly	3560	2844	2831
Propolis	2785	2025	1989
Roy./ Prop.	2492	2444	1914
Glucantime® 2%	3358	2046	2010
Glucantime® 4%	2537	2249	2252
Glucantime® 6%	2395	2076	1863
Glucantime®	2900	2450	1720

Table 6. (A) SI for amastigotes (CC₅₀/EC₅₀), (B) SI for promastigotes (CC₅₀/IC₅₀)

A			
Nano fiber\ Time	24 h	48 h	72 h
Royal J.	139.6	145	172.6
Propolis	111.8	99.7	113
Roy/Prop	73.3	116.4	109
Glucantime® 2%	49.5	57	85
Glucantime® 4%	67	76	96.6
Glucantime® 6%	37	102	127.8
Glucantime®	54.7	67.1	80.7

B			
Nano fiber\ Time	24 h	48 h	72 h
Royal J.	97	107.3	128.7
Propolis	58	61.4	56
Roy/Prop	31.5	87.3	81.5
Glucantime® 2%	21.3	33	34
Glucantime® 4%	14	18.4	56.3
Glucantime® 6%	11.6	32.4	43.7
Glucantime®	30.4	42.2	50.6

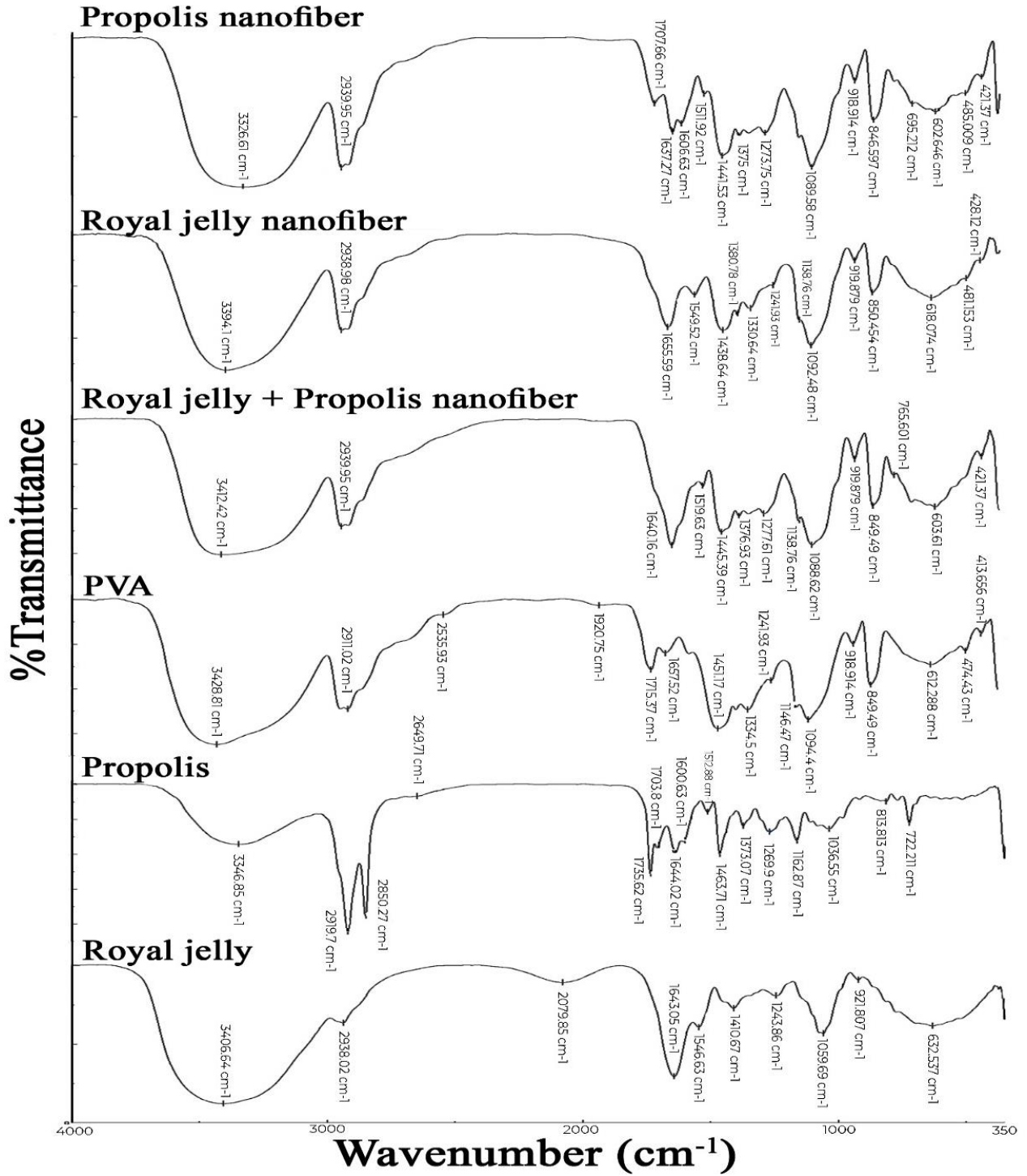


Fig. 3. FTIR spectra of different nanofibers compared to PVA, royal jelly, and propolis spectra (spectral range: 350-4000 cm⁻¹)



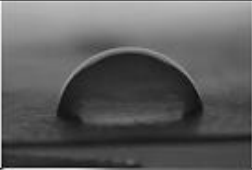
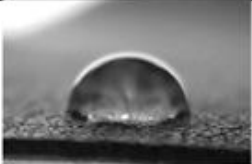
Analysis Result		
Sample	Contact angle	Picture
1	81±3	
2	84±6	
3	79±11	
4	87±8	

Fig. 4. Contact angle for electrospun samples: (1) Royal jelly Nanofibers (2) Propolis nanofibers (3) Royal jelly – Propolis Nanofibers (4) PVA

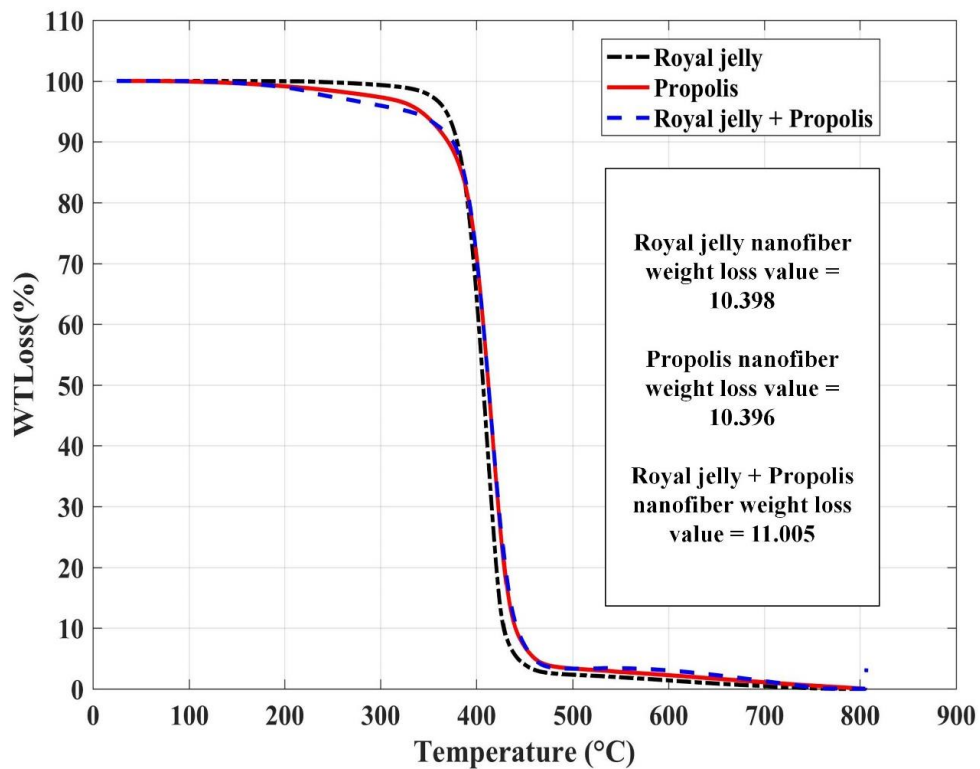


Fig. 5. Thermogravimetric (TGA) graphs of different electrospun nanofibers; Royal jelly, Propolis, Royal jelly-propolis combination

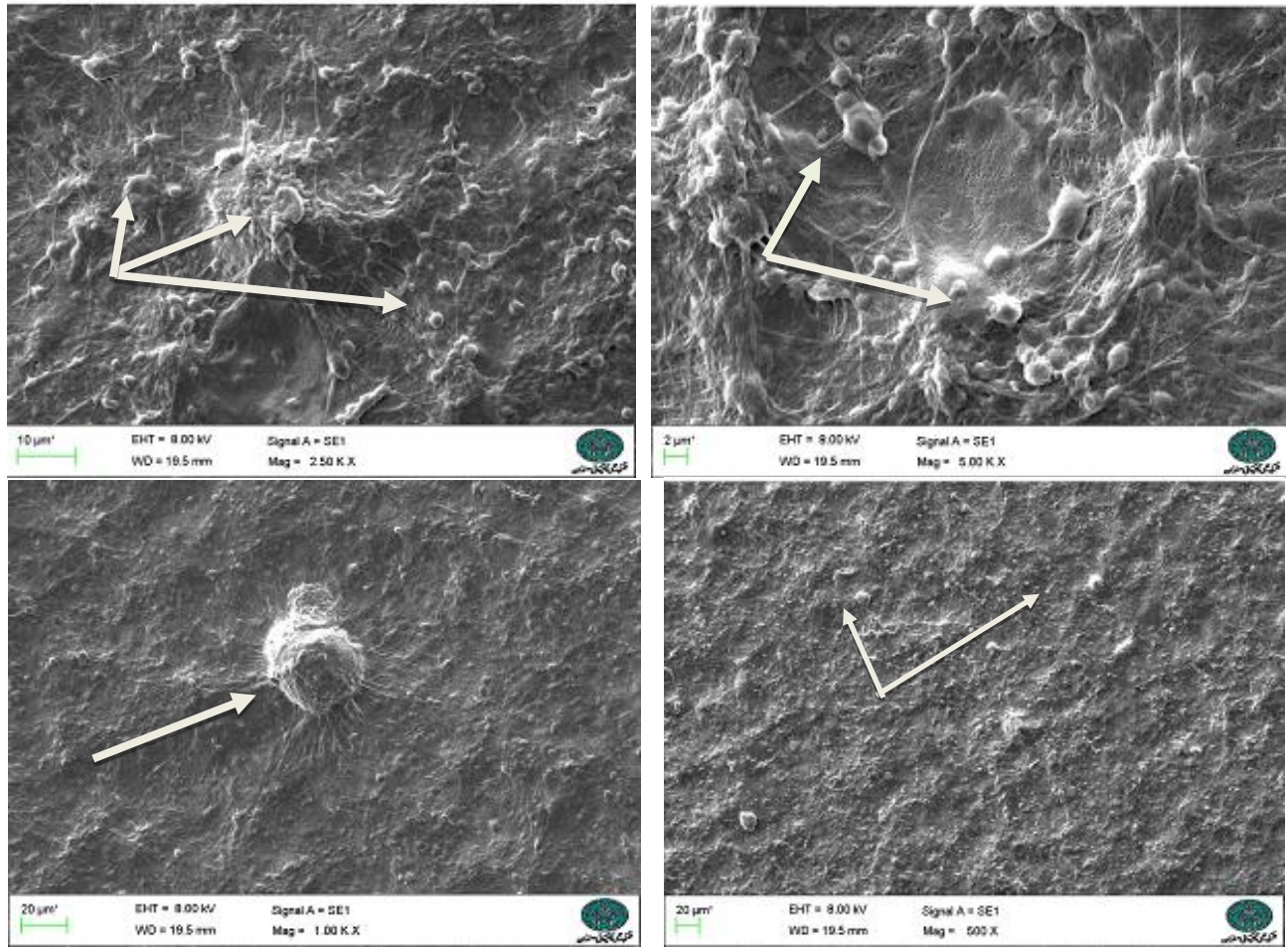


Fig. 6. Scanning Electron microscope Images (SEM) of macrophages on Royal jelly-Propolis nano-fibers for biocompatibility confirmation of nano-fiber. Viable macrophages surrounded by fibers can be seen

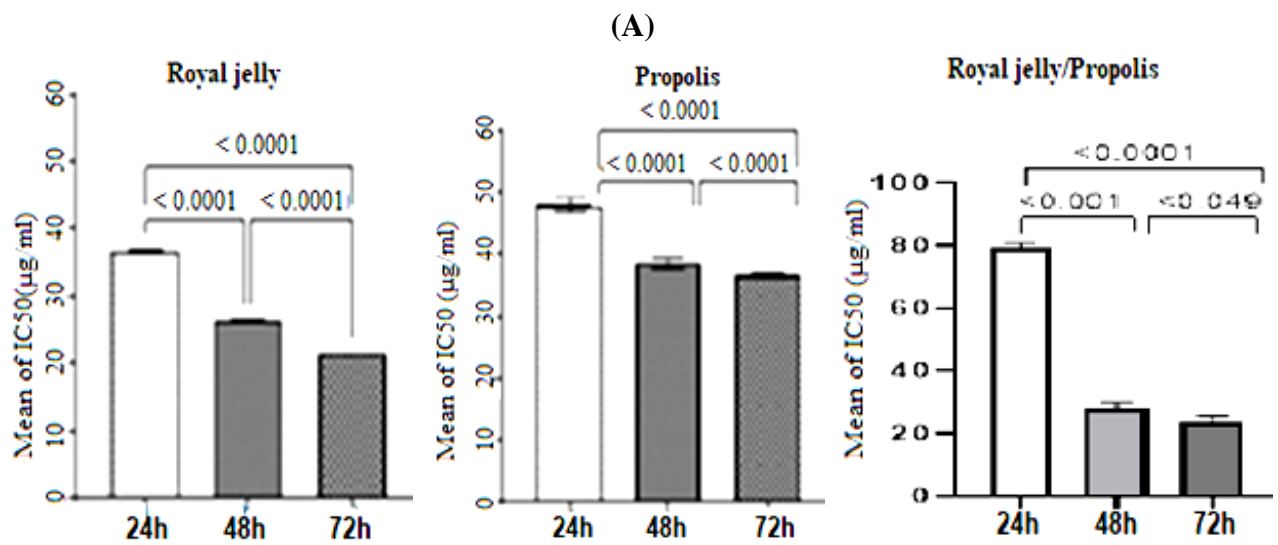


Fig. 7. Continued ...

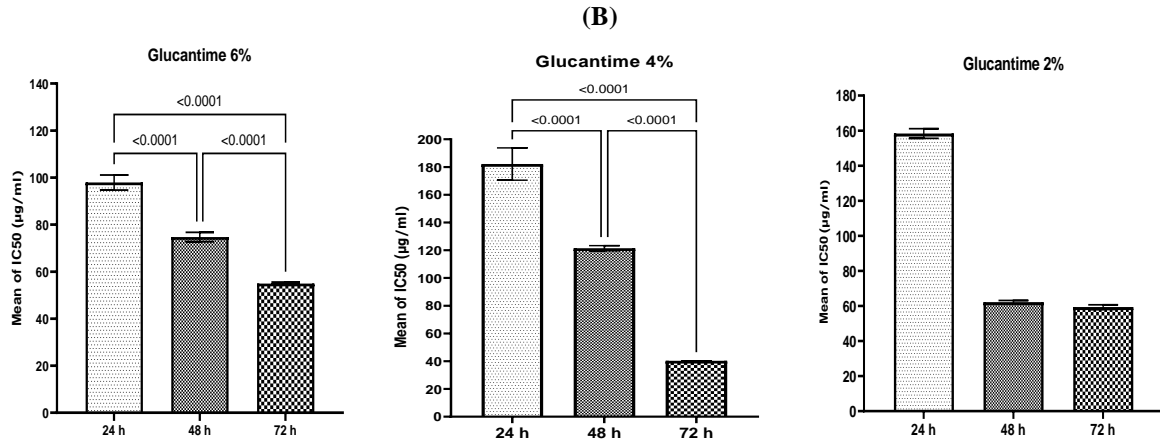


Fig. 7. Anti-Leishmanial effect (IC_{50}) of Glucantime® - free nanofibers (A) and nanofibers containing different concentrations of Glucantime® (The composition contains 2%, 4%, 6% Glucantime®) (B) in 24-48-72 h. A $p < 0.05$ is considered statistically significant

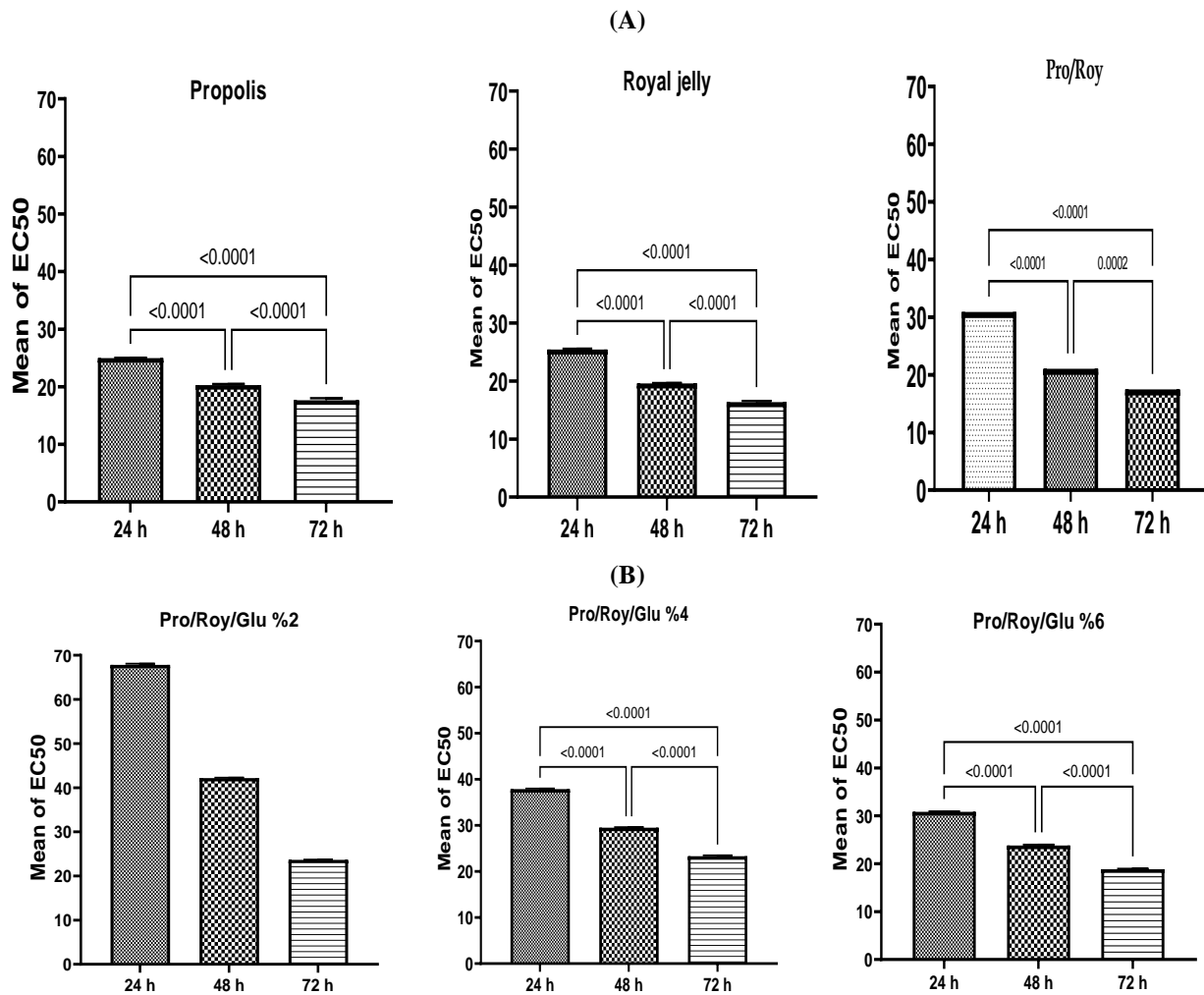


Fig. 8. Cytotoxicity assay (EC_{50}) of Glucantime® - free nanofibers (A) and nanofibers containing different concentrations of Glucantime® on *Leishmania major* amastigotes (B) in 24-48-72 h. A $P < 0.05$ is considered statistically significant

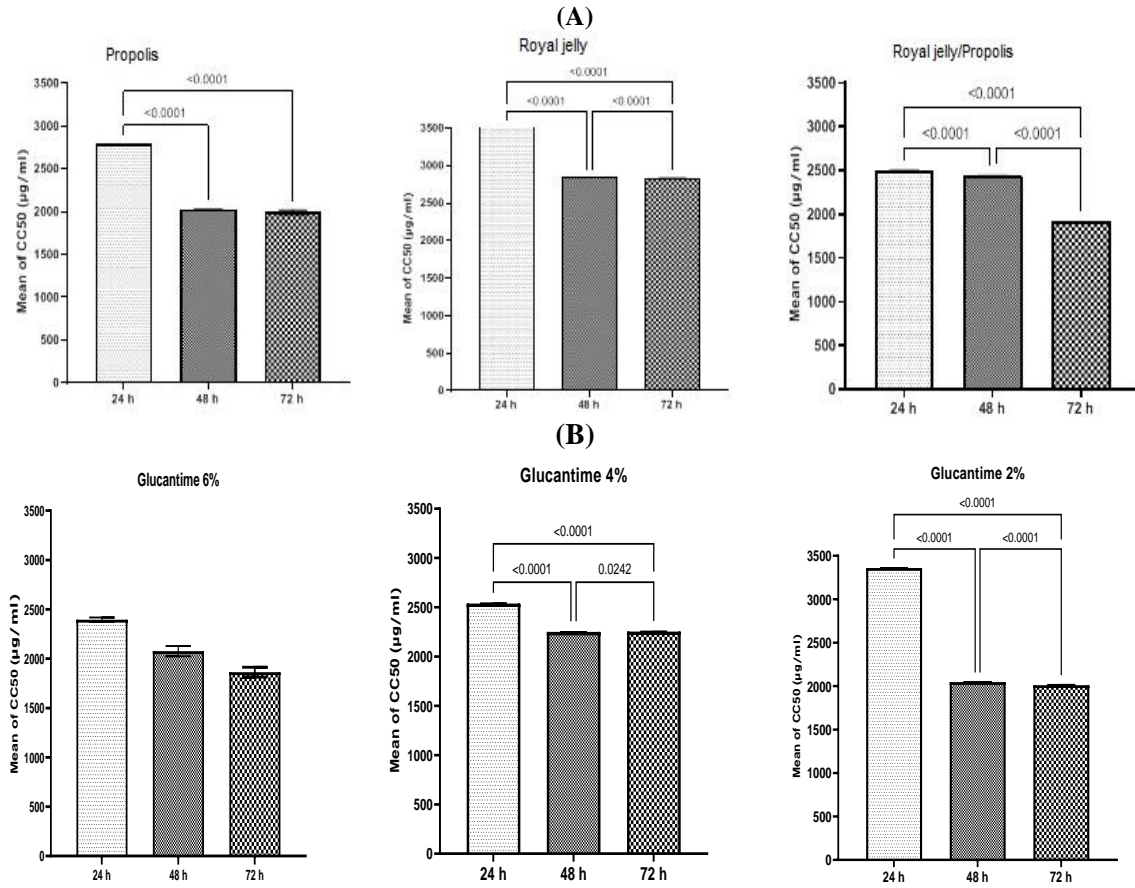


Fig. 9. Invitro cytotoxicity assessment of Glucantime® - free nanofibers (A) and nanofibers containing different concentrations of Glucantime® (The composition contains 2%, 4%, and 6% Glucantime®) (B) on macrophages in 24-48-72hours. A P < 0.05 is considered statistically significant

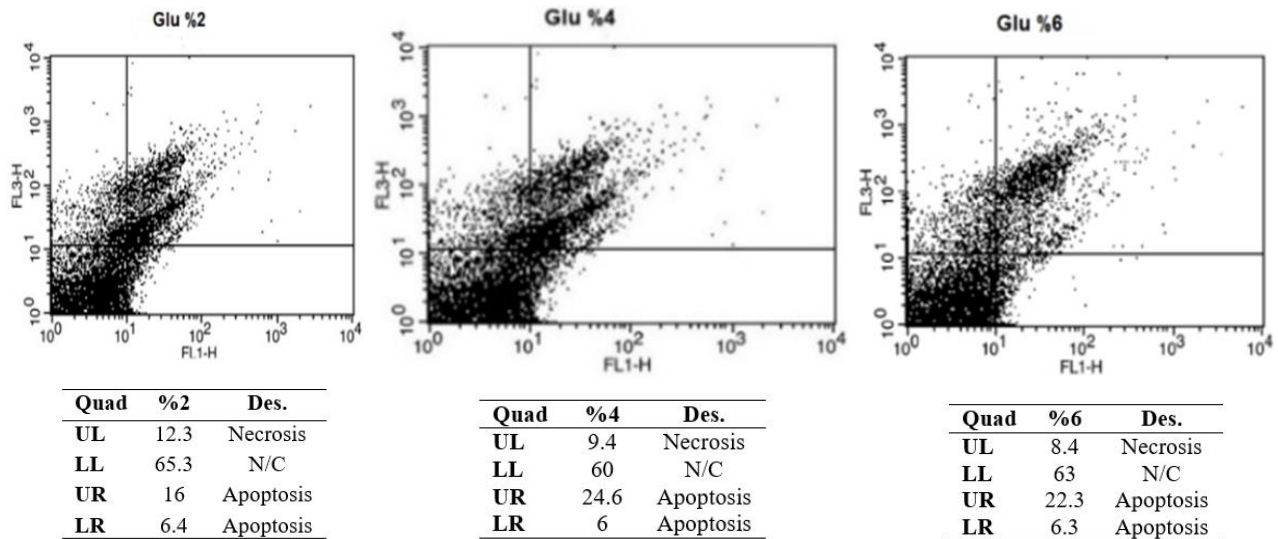


Fig. 10. Continued ...

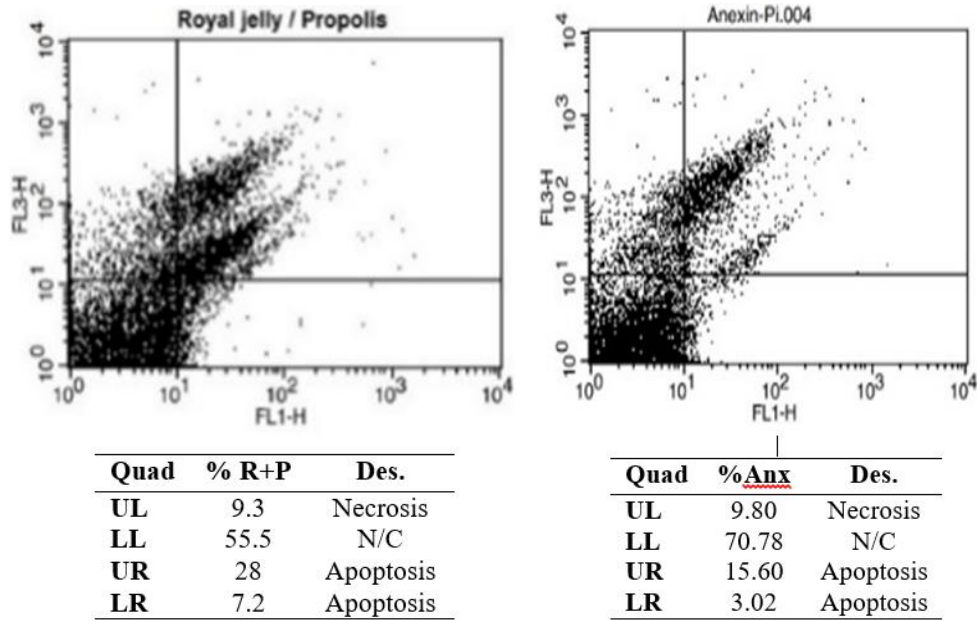


Fig. 10. Flow cytometry analysis of studied drugs in 24 hours

Discussion

Nowadays, researchers have endeavored to produce and to develop new and applicable drugs for treating various diseases by synthesizing nanomaterials, and they have not only succeeded in producing high-performance nano-drugs with minimal side effects but also in terms of biocompatibility, and reasonable price. Hence, in the present investigation, an attempt has been made to introduce a safe, effective, and natural drug to fight CL with minimal side effects. This research is a type of tissue engineering, in other words, we want to find a possible alternative way to cure the disease via the repair and reconstruction of damaged tissues. Several studies have confirmed the superiority of some natural or herbal medicinal products (simple or in nano form) on *L. major* compared to Glucantime®.

According to comprehensive literature review studies, no study has been published on the effect of Royal Jelly and Propolis nano-fibers on *L. major*. In line with this study, several reports confirm the antioxidant activity and significant therapeutic effects of propolis, and royal jelly on fungal, microbial, and parasitic

diseases. Brudzynski et al. (6) indicated that MRJP1 (Major royal jelly protein 1) in royal jelly is responsible for cell wall destruction of bacteria such as *Escherichia coli* (*E. coli*), *Bacillus subtilis* (*B. subtilis*). It is due to the presence of antimicrobial peptides: jellies 1, 2, 4. Zia et al. (44) demonstrated the significant inhibitory effect of propolis alcoholic extract on the growth of *Trichophyton mentagrophyte*, *Trichophyton rubrum*, and *Trichophyton verrucosum*. Diba et al. (45) confirmed the outstanding antifungal activity and inhibitory effect of propolis alcoholic extract on *Candida* and *Aspergillus* isolates in-vitro. Hamzepour et al. (46) showed that extracts of royal jelly and propolis, either alone or in combination, have an effectual inhibitory impact on the expression of the aflR gene in the production of aflatoxin in *Aspergillus parasiticus*. Mahmoudi et al. (47) showed the anti-leishmanial activity of propolis hydroalcoholic extract in the treatment of CL in BALB/c mice.

This study demonstrates the role of nano-based materials in enhancing their efficacy against leishmaniasis. This has been confirmed

by other studies. Goonoo et al. (48) claimed to find nanomedicine-based approaches to address cutaneous lesions given obtaining scarless wound healing, targeting secondary bacterial infection caused by *L. major*, reducing drug cytotoxicity, preventing parasite transmission and relapse. Torabi et al. (49) have demonstrated the efficacy of Nanogold for the treatment of *L. major* (with methanol Extract of *Eucalyptus camaldulensis*) on BALB/c. They showed that the amastigote number in the lesions was significantly reduced in interventional groups compared with control groups. Nanogold solutions also lessened the mortality rate in the mice. Mohebbi et al. (50), confirmed the inhibitory effect of nanosilver on *L. major* in-vitro and in vivo. Mohtasabi et al. (51) investigated the anti-leishmanial effects of biogenic antimony sulfide nanoparticles (NPs) on *L. major* in both in-vitro and in vivo experiments. They showed that the drug in the form of nanoparticles significantly reduced the parasite load.

In recent years, in addition to the aforementioned substances, increasing attention has been paid to other natural products and alternative herbal compounds for the treatment of leishmaniasis. Many studies confirm the antileishmanial activity of these compounds. Gholamrezaei et al. (52) have shown the significant inhibitory effect of *Foeniculum vulgare* aqueous and alcoholic seed extract against *L. major*. Badirzadeh et al. (53) revealed that *Urtica dioica* extract treats the *L. major* by switching the mouse immune response towards a cell-mediated response. Najm et al. (54) claimed that ethanolic extracts of three species of *Artemisia*, including *A. persica*, *A. spicigera*, and *A. fragrans* are potent, influential, and safe herbal compounds for removing the *L. major* with less cytotoxicity.

Since the combined nanofibers contain antimony metal, ICP-OES (Inductively coupled plasma-optical emission spectrometry) was carried out to investigate the drug release rate and Kinetic of the antimony from the nanofibers scaffold at different times. For this purpose, the

nanofibers containing Glucantime® 6% were used (18 µg/ml of pure antimony). According to the obtained results (Table 2), about 64% of drug-release occurred within 48 hours. Hence, antimony was steadily released from the nano-scaffold, and the maximum released amounts occurred in the early hours. The most significant portions of drugs seeped out of the nanostructure in the first 24 h (more than half of the loaded drug) and continued in the following hours. Therefore, the parasite should be exposed to the nano-drugs for at least 24 hours. Different values of IC₅₀ and CC₅₀ were obtained based on time intervals by MTT assay.

Inhibitory effect of the nano-drug on Promastigotes (IC₅₀)

Compared to Glucantime® free nanofibers, royal jelly nanofiber significantly showed an excellent effect on promastigotes (IC₅₀= 35 µg/ml) in the first 24 h, followed by propolis (IC₅₀= 48 µg/ml) (P< 0.05), but the impact of both above nanofibers was significantly higher than pure Glucantime® (IC₅₀= 95.3) (P< 0.05). Nevertheless, the inhibitory effect of the proposed drugs was time-dependent, and there is a direct relationship between the exposure time and the nanofibers' effectiveness (IC₅₀). Maximum activity is observed after 72 hours. IC₅₀ of the combined nanofibers (Royal jelly+ Propolis) dropped from 79 µg/ml to 23.5 µg/ml after 72 hours (Fig. 7A).

Regarding combined containing different concentrations of Glucantime® (2%, 4%, 6%) on *L. major* (Fig. 7b), in the first 24 h of incubation, the combinations plus 6% Glucantime® with an IC₅₀ of 98 µg/ml statistically had greater anti-leishmanial activity than the other two concentrations. But after 72 h, the compound plus 4% Glucantime® showed the highest inhibitory activity compared to other compounds (IC₅₀= 40 µg/ml). In an overview, the inhibitory activity (IC₅₀) of royal jelly is significantly better than other studied drugs (either Glucantime® -free or containing Glucantime® nanofibers) at all times, even Glucantime® alone. It is worth noting that most of the proposed drugs

have statistically shown greater anti-*leishmanial* activity than Glucantime® as standard ($P < 0.05$).

In-vitro Toxicity evaluation of nanofibers on amastigote forms (EC₅₀)

As shown in Fig. 8A, the lethal activity of royal jelly nanofibers against *L. major* amastigotes (EC₅₀= 16.4 µg/ml) was significantly better than other nanofibers at different time intervals, followed by nanofibers of propolis and combined drugs (EC₅₀= 17.5 µg/ml). These outcomes have been significantly better than similar researches (35, 44, 49). The findings show the statistically significant inhibitory activity of all Glucantime®- free on amastigote forms ($P < 0.05$).

Regarding the combinations containing Glucantime® (Fig. 8B), the 6% combination revealed the most significant inhibitory effect on amastigotes at different times. Having regarded the incremental release of the drug from the scaffold, its inhibitory effect has enhanced over time (30.8 µg/ml, 23.8 µg/ml and 18.8 µg/ml respectively). There are slight differences in the effectiveness of the 4% and 6% combinations. In general, the effectiveness of the studied nanofibers has increased gradually after 24 hours. Overall, we found that royal jelly nanofibers and formulations containing 6% Glucantime® had the greatest inhibitory effect on amastigotes (the efficiency of royal jelly was significantly higher). As seen, the drug efficacy has gradually augmented after 24 hours.

In-vitro Cytotoxicity evaluation of nanofibers on macrophages

As shown in Fig. 9A, compare to other nanofibers, royal jelly had significantly lowest macrophage cytotoxicity at any given time ($P < 0.05$), while the highest corresponding to combined with 6% Glucantime®. As shown in Fig. 10, compared to other nano-structures, royal jelly nanofibers had significantly the lowest macrophage cytotoxicity at any given time ($P < 0.05$). At the same time, the highest was corresponding to the combination of 6% Glucantime® (Propolis contains many chemical compounds

such as phenolic acids, aromatic compounds, and flavonoids including flavones, flavonones, quercetin, etc., which may play an essential role in its antimicrobial activity). However, the cytotoxicity of the six proposed nanofibers was very slight and negligible. The results of the present study were significantly better than similar studies (35, 44, 49). Cytotoxicity of six studied drugs was gradually increased within 72 hours. For the royal jelly/propolis combination, the incremental slope was very gentle.

Compared to the proposed nanomedicines, the 72 h anti-leishmanial selective values of royal jelly and Glucantim® 6% composition were the highest (172.5 µg/ml and 127.8 µg/ml, respectively) against intracellular amastigotes (CC₅₀/EC₅₀). Moreover, SI was calculated for promastigote forms (CC₅₀/IC₅₀). As can be noticed in the tables (Table 6), after 72 h of incubation, the highest SI value was concerned with royal jelly nanofibers and the simple combined drugs (royal jelly+ propolis) (128.7 µg/ml and 81.44 µg/ml respectively).

Flow cytometry analysis

According to the findings (Fig. 10), we realized that the cell death either induced by the proposed nano-medicines, through programmed cell death mechanism (PCD) or through cell necrosis, was significant. Having regarded the importance of health maintenance of adjacent cells after cell death (due to releasing many chemicals from the dying cells), the mechanism of apoptosis is more favorable than cell necrosis. Among the studied drugs, the drug in combination (Royal jelly/Propolis) had the highest rate of apoptosis in the early and late stages (7.13%, 28.9% respectively) compared to the control group (3.02%, 15.6%) ($P < 0.05$). In the combination group containing Glucantime®, the total lethality rate of the combined nanofibers plus 4% Glucantime® was 40.02%, which was the highest rate among the rest of the compounds in this group, and more than 30% of it was associated with cell apoptosis. The most increased cell necrosis pertains to the 2% compound (12.3%). In this regard, among the com-

pounds containing Glucantime®, the combination plus 4% is more acceptable. The results of SEM, TEM, FTIR, Contact angle, and TGA demonstrate that the proposed nanofibers pose an appropriate structure and function for therapeutic goals and all the features of a suitable coating for the treatment of *L. major* lesions.

Conclusion

The findings of this study showed that royal jelly and propolis nanofibers, either individually or in combination, are significantly capable of inhibiting the growth of *L. major* with minimal cytotoxicity. In-vivo studies are needed in the future. The importance of combined is due to the simultaneous presence of propolis and royal jelly in the combination and the remarkable antimicrobial role of propolis. The continuation of this study in the In vivo phase is ongoing. It is suggested to do more studies on similar nanostructure drugs to cure other infectious and non-infectious skin diseases.

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Ethical Consideration

The Ethical Committee of the Faculty of Medicine of Iran University of Medical Sciences has approved the procedures required for the study. Code No: IR.IUMS.FMD.REC.1399.511.

Conflict of interest statement

The authors declare there is no conflict of interests.

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