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Therapeutic Effects of the As-Synthesized Polylactic acid/Chitosan Nanofibers Decorated with Amphotricin B for *in vitro* Treatment of *Leishmaniasis*

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Abstract

Leishmaniasis as a third most important vector-borne disease caused by different species of a *Leishmania* flagellum protozoan. *Leishmaniasis* as a one of the biggest concerns of the World Health Organization due to the severe side effects, the emergence of resistance, secondary bacterial infections, reports as epidemics especially in immunocompromised individuals. The main purpose of this study was synthesis and characterization of the polylactic acid/chitosan nanofibers decorated with amphotricin B as a new delivery system for in vitro treatment of *Leishmania* wounds. The prepared nanofibers were characterized by scanning electron microscopy (SEM), Fourier transform infrared spectroscopy (FT-IR), Dynamic light scattering (DLS), X-ray diffraction (XRD) and in vitro drug release test. The *anti leishmaniasis* effect of nanofibers decorated with amphotricin B against *Leishmania major* promastigotes and its cytotoxicity on macrophages were determined respectively with flow cytometry test. The *in-vitro* drug release assay indicates 84% of the amphotricin B loaded in nanofibers after 400 minutes. The average concentration of the amphotricin B loaded in polylactic acid/chitosan nanofibers and conventional form of amphotricin B that prevented the growth of 50% of the promastigotes of L.*major* was 1.29 and 4.34 µg/ml, respectively.

Keywords: *Anti leishmanial* effects, *Lishmania major*, Nanofibers, Drug delivery

1. Introduction

Leishmaniasis as a third most important vector-borne disease with an important clinical and epidemiological diversity caused by different species of a *Leishmania* flagellum protozoan[1, 2]. According to the World Health Organization (WHO), *leishmaniasis* as a major public health problem is one of the seven most important tropical diseases due to its wide spectrum of clinical manifestations [3], [4, 5]. *Leishmaniasis* is a sophisticated disease for both humans and animals [6, 7]. Cutaneous manifestation include localised cutaneous leishmaniasis [LCL] (ulcerative skin lesions) and diffuse cutaneous leishmaniasis [DCL] (multiple non-ulcerative nodules) [5, 8, 9]. Amphotericin B as a macrolide polyene antifungal antibiotic agent has leishmanicidal and Antibacterial activity. It is the best alternative in patients that showed resistance to treatment with antimonials compounds[10, 11]. In recent years, new NPs are specifically considered in treatment of leishmaniasis (Table 1). The nanofibers as a classification of widely used nanostructures have an efficient drug delivery according to their high surface area to volume ratio, adjustable drug release profile, possibility of controlling the crystalline-amorphous phase transition, high porosity and malleability[12]. Novel wound dressing due to their resemblance to the skin structure and extracellular matrix (ECM) are one of the reasons that they can be use as drug delivery systems [13-15]. Thus, release control can be achieved using fiber-based formulations and several types of drugs and bioactive agents can be incorporated directly into the core[16], including antibiotic[17], antineoplastics[18], analgesics[19], non-steroidal anti-inflammatory[20], antimicrobials[21], antifungals, DNA, proteins or growth factors [21, 22]. Chitosan nanoparticles due to their antioxidant activity are a good option for improving resistance to microbial infections [22-24]. Although some disadvantages such as limited solubility in most organic solvents, three-dimensional networks of strong hydrogen bonds[25]and high molecular weight have set up

obstacles along the way of electrospinning of the chitosan [26, 27]. Polylactic acid due to its excellent properties such as non-toxic, strong mechanical strength and good fiber forming ability was chosen as a co-material for electrospinning to enhance the electrospinning ability of chitosan and improve mechanical strength of the nanofibers[28]. In this study therapeutic effects of the as-synthesized polylactic acid/chitosan nanofibers decorated with amphotricin B for *in vitro* treatment of *Leishmaniasis*. The size, morphology, chemical bonds and thermal stability of the final products were investigated with SEM, FT-IR, DLS and TGA.

Table1. Some of researches for application of nanostructures on *Leishmania* in recent years.

Nanostructures	Method	Leishmania species	Stage employed	Reference
Lipid NPs	emulsion	L. infantum	Promastigote	[29]
Ag NPs	solution	L.tropica	Promastigote	[30]
ZnO	Green synthesis	L. major	Promastigote	[31]
Au NPs	plasmon response	L. donovini	Amastigote 540 nm	[32]
Fe_2O_3	hyperthermia	L. mexicana	Amastigote	[33]

2. Experimental

2.1. Materials and methods

All reagents were used without further purification and purchased from authorized dealers. [$(C_{16}H_{33})$ $N(CH_3)_3$ Br, cetyltrimethylammonium bromide ≥98%, micellar avg mol wt 62,000], N, N Dimethylmethanamide, $HCON(CH_3)_2$, MW: 73.09, 99.8%, Polylactic acid (PLA) granule, (MW = 60,000 g/mol) with purity >99% and 3 mm nominal granule size powder were purchased from Sigma-Aldrich Company. Chitosan with a molecular weight of 690,000 g/mol and deacetylation degree of 90% was supplied by Biomaterials Co. (Korea). The drug used is Amphotericin B

(Sigma–Aldrich). All the aqueous solutions were prepared by using Deionized water (DW) which was purchased from the ABSAN laboratory.

2.2. Preparation of electrospinning solutions

In this study, first of all we synthesized and prepared amphotericin B nanoemulsions. Then, amphotericin B nanoemulsions were decorated in PLA/chitosan nanofibers as biodegradable and bioactive compounds. For this purpose, certain amount of sodium dodecyl sulfate (CTAB) as surfactant phase dissolved in 20 ml deionized water under vigorous stirring with 600 rpm at 45 ºC for 120 min up to obtained critical point (CMC). Then, 50 mg amphotericin B was dissolved in 10 ml of DMF under reflux system at 600 rpm and 45 ºC for 45 min. In the final step solution was exposed to microwave irradiation at 300 watts for 5 minutes with periods of 30 seconds on and one minute off to the preparation of amphotericin B nanoemulsion. PLA/chitosan nanofibers were prepared by the electrospinning method. Electrospinning device was set up with a high-voltage power supplying generating voltages up to 30 kV, as the source of the electric field. The feed rate of the sprayed polymer on the controller was 1.0 mL/h with electrical potential 15 kV. For this purpose, 5.0 g PLA powder was dissolved into 5 mL DMF and stirring at 50 °C for 30 min. Then a certain amount (10 wt%) chitosan was added to this solution under the ultrasonic waves 60 watt for 45 minutes. Then 5 ml of amphotericin B nanoemulsion added to the above solution and pH was adjusted between 7.1-7.8. In the across process, the distance between the tip of the nozzle and the outer surface of the drum was 10 cm. Finally, nanofibers were collected on a sheet of aluminium, the system was allowed to cool to room temperature naturally, and the obtained precipitations were collected[34, 35].

2.3. Leishmania major parasites and macrophage cells

Leishmania major promastigotes were cultured in RPMI-1640 medium, 10% heat-inactivated fetal bovine serum (FBS) and 1 µl penicillin-streptomycin (10,000 IU/ml) at 24 ºC. Murine macrophage cells J774 were obtained from the Pasteur Institute of IRAN. The macrophages were placed in a flask and cultured in DMEM, 15% inactivated fetal bovine serum (FBS) and 1 µl penicillin-streptomycin (10,000 IU/ml) in an incubator at 37 ºC with 5% CO_2. Fresh RPMI-1640 medium plus 10% FBS was added to the flask after removing the supernatant of the cells daily under sterile conditions[36].

2.4. In vitro drug release

In order to simulate skin permeation condition, *in vitro* release study was performed using a Franz diffusion cell [37]. In this study, cellulose-acetate filters with the pores size of 45 µm were used due to its resemblance to skin and mounted between the receptor and donor compartment of the diffusion cell [38, 39]. *In vitro* amphotericin B release from PLA/chitosan nanofibers was studied with UV spectrometer S-3100 SCINCO in 0–400 min within the wavelength range of 200nm to 800nm against ethanol as blank, maximum absorption peak at 383 nm and 408 nm.

2.5. Antioxidant assay

To evaluate the antioxidant effect of nanofibers, 1 mL of different concentrations (6.25, 12.5, 16, 25, 50, 100 and 200 µg/ml) of PLA/chitosan nanofibers mixed with 1 ml of DPPH solution (2,2-diphenyl-1-picrylhydrazyl) and dissolve in methanol. 3 mL of methanol added again to the reaction mixture. The mixture was kept in a dark place for 20 minutes. Then, the light absorption of each samples was measured at 490 nm and the control was prepared by deionized water. BHA was used as a positive control, which is a synthetic antioxidant. The percentage of antioxidants was also evaluated according to Eq.1.

$$DPPH\ radical\ scavenging\ ability\ (\%) = [1 - (A - B)/C] \times 100 \quad \text{Eq.1}$$

which A states the adsorption of samples mixed with DPPH and B states the adsorption of samples without DPPH and C is the control adsorption. The concentration required to inhibit 50% of DPPH (IC_{50}) is calculated using a linear regression model.

2.6. Evaluation of drug toxicity to macrophages

In order for these devices to have medical applications, they should be harmless to human cells and organs. The cytotoxicity of Polylactic acid/Chitosan nanofibers decorated with amphotricin B were investigated on macrophages using Flow cytometry (BD FACSCalibur™, San Jose, California, USA). To flow cytometry cytotoxicity assay 1 mL of macrophages at a final concentration of 10^6 cells/ml were counted under a microscope and plated in a 6-well microplate and incubated at 37 ºC in a CO_2 incubator for 24 h. After the incubation, 100 µL of Polylactic acid/Chitosan nanofibers containing different concentrations of amphotericin B were added to the macrophages and the volume was increased to 1 mL with culture medium. Then the cells were incubated again at 37 ºC in a CO_2 incubator for 72 h. After that, the cells were scrapped and transferred to 0.5-mL microtubes and centrifuged to settle the macrophages. Then they were washed twice with the cold PBS solution and 1 mL of binding buffer was added to them. Finally, 100 µL of the whole mixture was removed and 5 µL of annexin V-IFTC and 5 µL of 7-aminoactinomycin D (AAD7) were added to it and the mixture was subsequently evaluated by a flow cytometer[6, 40].

2.7. Flow cytometry apoptosis assay

Since the main purpose of this study was to synthesize amphotericin B loaded nanofibers to treat cutaneous *Leishmaniasis*, the efficiency of different concentrations of nanofibers towards the parasites was investigated. The apoptotic, necrotic, and viable cells percentage for each sample was determined by analyzing the flow cytometry diagrams using FlowJo software. For this

purpose, 10^6 promastigotes/ml of L. *major* were cultured in RPMI medium containing 10% (v/v) fetal bovine serum (FBS-Serum, Germany) at 25 °C with 5% CO_2. According to the kit instructions, the parasites were stained by annexin V-IFTC and 7AAD and analyzed using a Becton Dickinson flow cytometer. To perform this assay, first, the test specimens containing about one million parasites per ml of total reaction volume and 100 µL of different concentrations of amphotericin B and PLA/chitosan nanofibers containing different concentrations of amphotericin B were added to sterile microtubes. The microtubes were centrifuged at 14,000g for 15 min, and then the supernatant was discarded and the sediments in all the microtubes were washed twice with the cold PBS solution. The binding buffer solution was added to the washed precipitate and kept in a dark place at 26 ºC for 20 min. Finally, antibodies were added to each microtube, mixed well, and tested using a flow cytometer (BD FACSCalibur, San Jose, California, USA)[36, 40].

2.8. Statistical analyses

Statistical data analyses were performed using two-way analysis of variance (ANOVA) and *t*-test in the SPSS software ver. 20 (Chicago, Illinois, USA). IC_{50} and CC_{50} were measured by probit analysis in SPSS software and the results were considered significant at $P \leq 0.0001$.

3. Resaults and Discussion

3.1. Characterization

The morphology and size were evaluated using scanning electron microscopy (LEO 1455VP, Leo Trading Company, Hong Kong) with an accelerating voltage of 20 kV. The results of SEM images of the Polylactic acid/Chitosan nanofibers (with the magnification of 200 nm), nanofibers decorated with 50 mg amphotricin B (with the magnification of 200nm and 100 nm) and nanofibers decorated with 100 mg amphotricin B nanoemulsion are shown in Figure 1a-1d. nanofiber. As can be seen from the results the effect of the amphotricin B on the morphological

and surface properties of the Polylactic acid/chitosan nanofibers is clearly traceable. It can be seen that in the absence of amphotricin B, nanofibers structures were synthesized without any tangles and rupture (Figure 1a), when amphotricin B was added to nanofibers structures, it creates sites on the fibers which are related to decorated amphotricin B. Figure 1b and Figure 1c show this phenomenon with scales 200nm and 100nm respectively. After adding 100 mg amphotricin B to nanofibers structures, SEM images show high agglomeration was obtained which this can be due to increase the surface-to-volume ratio at amphotricin B nanoemulsions (Figure 1d).

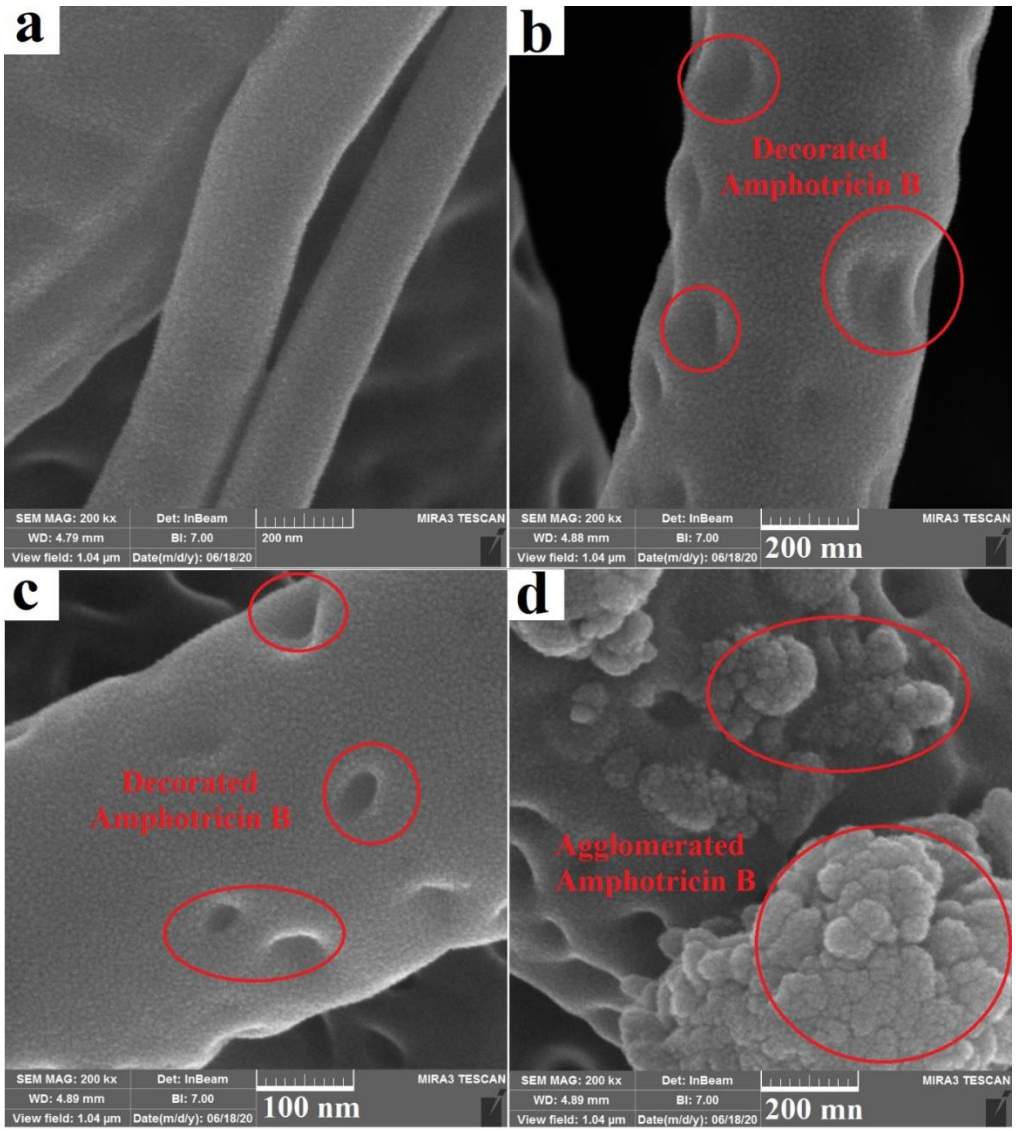


Figure 1. SEM image of PLA/chitosan nanofibers (a) PLA/chitosan nanofibers decorated with 50 mg amphotricin B with the magnification 200nm (b), with the magnification 100 nm (c), PLA/chitosan nanofibers decorated with 100 mg amphotricin B (d).

The hydrodynamic diameter and size distribution measured with dynamic light scattering (DLS) analysis (Mastersizer 2000E, Malvern Instruments, UK). The particle size of the Polylactic acid/Chitosan nanofibers and Polylactic acid/chitosan nanofibers decorated with amphotericin B were analyzed with DLS. According to the DLS data analysis, Dn 10%:112.77 nm Dn 50%:129.55 nm Dn 90%:155.87 nm were obtained for nanofibers and Dn 10%: 89.5 nm Dn 50%: 107.68 nm

Dn 90%: 431.06 nm were estimated for nanofibers decorated with amphotericin B. As can be estimated from the results it can be seen that by loading amphotericin B nanoemulsions in nanofibers their size will be larger. DLS data analysis for the Polylactic acid/Chitosan nanofibers and Polylactic acid/chitosan nanofibers decorated with amphotericin B are showen in Figure2a and 2b

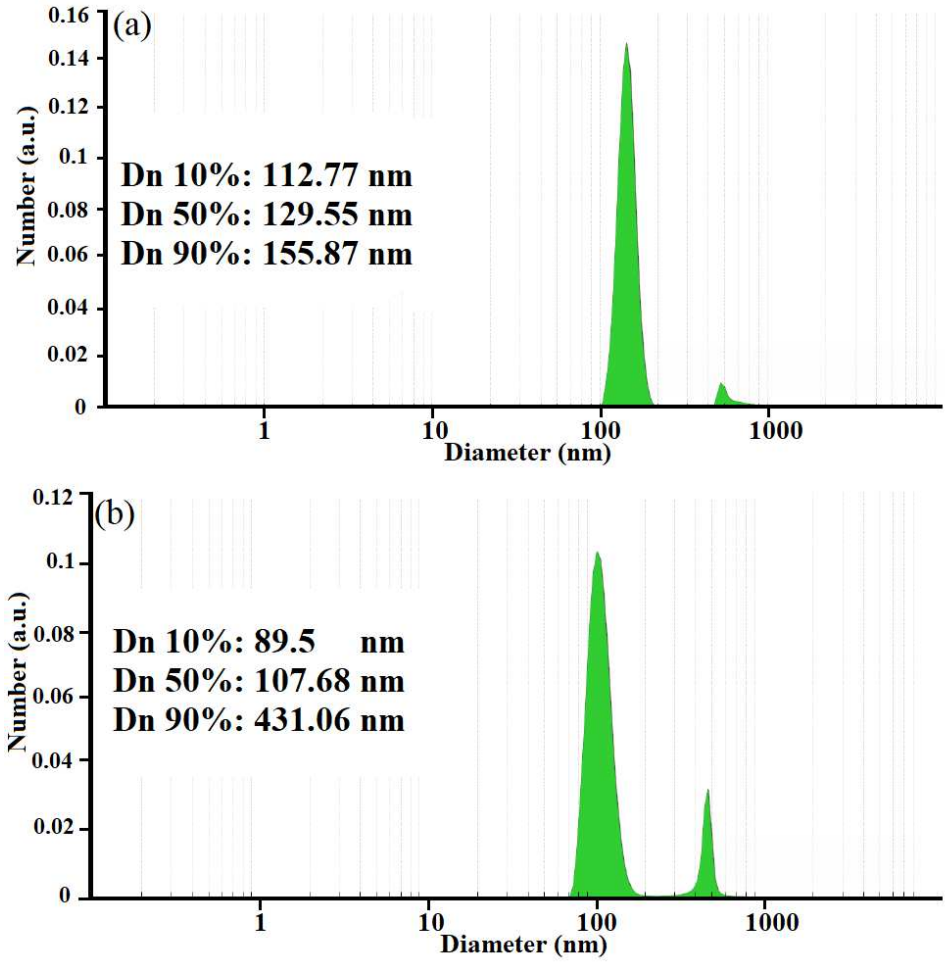
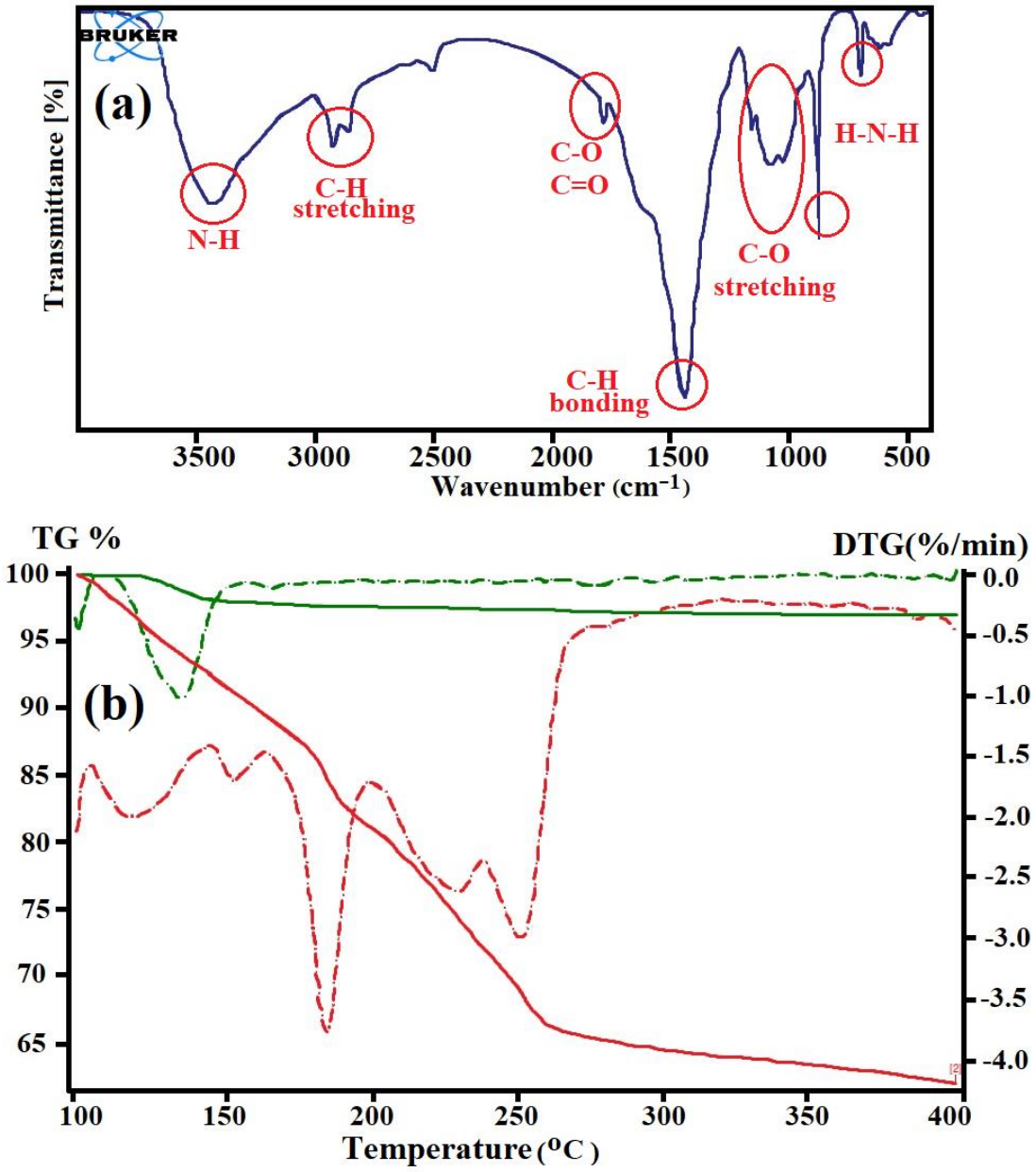


Figure 2. DLS data analysis for the Polylactic acid/Chitosan nanofibers and Polylactic acid/chitosan nanofibers decorated with amphotericin B.

Functional groups and chemical composition of the nanofibers decorated with amphotericin B were characterized FT-IR spectroscopy (Tensor II, Bruker, Germany). The FT-IR spectroscopy of

Polylactic acid/chitosan nanofibers decorated with amphotericin B is shown in Figures 3a. The FT-IR analysis peaks were measured in the range of 500 cm^{-1} to 3500 cm^{-1}. The peaks around 3450 cm^{-1} can be related to N-H groups in amphotericin B and chitosan molecules. The spectral reflectance near 950 cm^{-1} ascribed to H-N-H group vibrations and C-N stretch band. As can be seen in the IR peaks of C-H stretching and bonding CH_2 groups has became obvious at 2964 and 1480 cm^{-1}. Results of FT-IR spectroscopy show, preparation of Polylactic acid/chitosan nanofibers confirmed by literatures[41]. Thermogravimetric analysis (TG) represents a curve for weight change as a function of temperature or time. Figures 3b shows the TGA profile for the Polylactic acid/chitosan nanofibers decorated with amphotericin B under the nitrogen atmosphere at a heating rate of 10 °C/min. As can be seen the TG curve exhibits a one stage of decomposition for the final product. Due to the formation of nanoemulsion structures and hydrogen bonds slight thermal stability of the formulation is observed and thermal degradation occurs rapidly. It is anticipated which at the first the cross linking between amphotericin B and Polylactic acid/chitosan are destroyed at 130°C, after this temperature, more mass of the final product lost, this can be related to the complete thermal decomposition of the Polylactic acid/chitosan nanofibers.



233

234 **Figure 3.** FT-IR spectroscopy of Polylactic acid/chitosan nanofibers decorated with amphotericin

235 B (a) and TGA profile for the Polylactic acid/chitosan nanofibers decorated with amphotericin B.

236

237 For *in vitro* drug release estimated five points standardization curve were obtained in a

238 concentration range from 0.312 to 5µg/ml for amphotericin B. The response of the drug was found

239 to be linear within the investigation concentration range and the linear regression equation

240 calculated y=0.1376x+0.0021 with correlation coefficient R=0.999. Figure 4a and 4b show
241 calibration curve and the cumulative release profiles of amphotericin B polylactic acid/chitosan
242 nanofibers ($p \leq 0.005$) respectively. Results show 84% of the amphotericin B were released after
243 400 min from nanofibers. Intertwined polymer structures of the polylactic acid/chitosan nanofibers
244 leads to more drug trapped in network structures and Causes a continuous release of the drug.
245 Insertion of the drug into the nanofibers resulted in the release of less than 60% of loaded drug
246 within 2 h instead of sudden drug release. The burst release for Amphotricin B is very low at the
247 initial moment of the process and then in the first 30 minutes, drug release reaches to 20%.

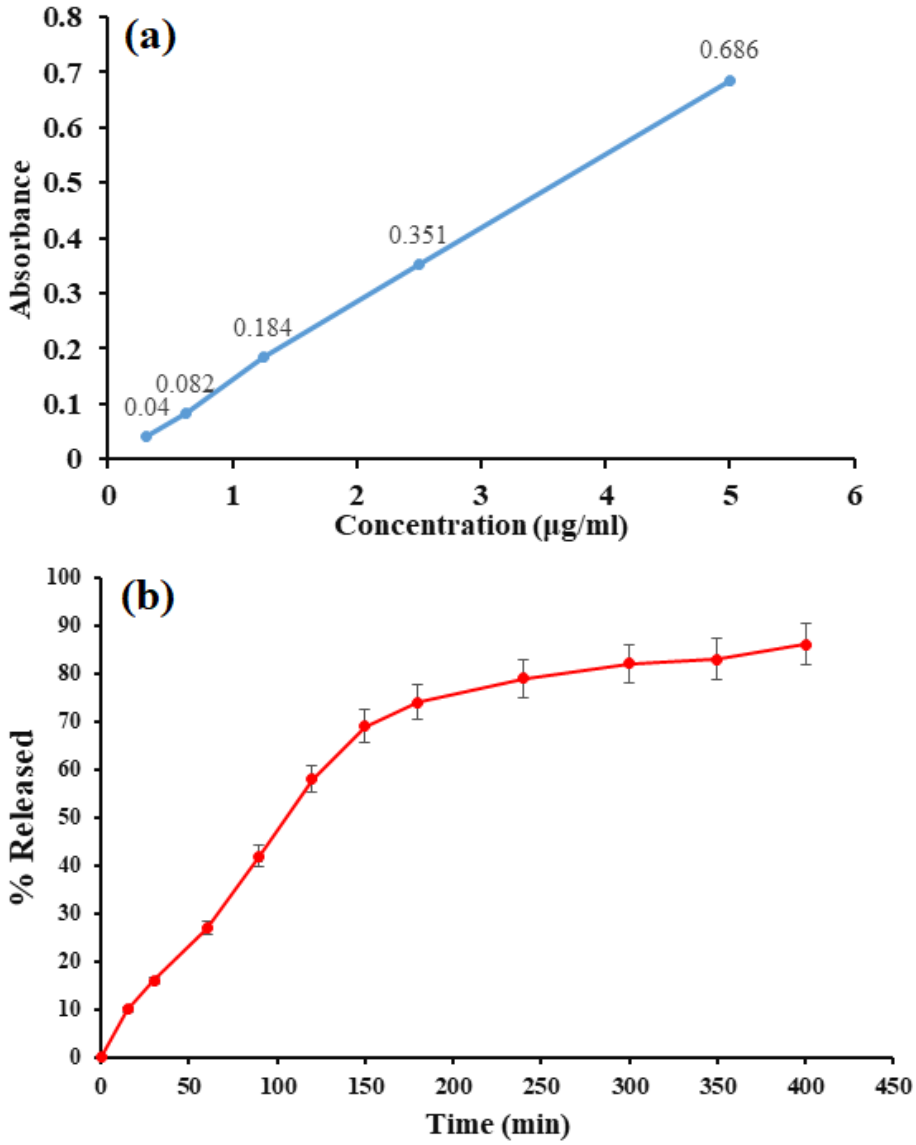


Figure 4. Concentration vs absorbance table for linearity study of amphotericin B (a), The cumulative release profiles of Amp B from core/shell nanofibers (Mean ± SD, n=3) (b).

Briefly, to analysis the radical-scavenging activity of PLA/chitosan nanofibers and BHA on DPPH, assessing the hydrogen donations from these compounds was examined. According to figure 5, the overall IC_{50} value of BHA and PLA/ chitosan nanofibers was 38.11 µg/ml and 60.34 µg /ml respectively. Statistical analysis showed a difference between PLA/ chitosan nanofibers and BHA, although the antioxidant activity of nanofibers was still in acceptable range.

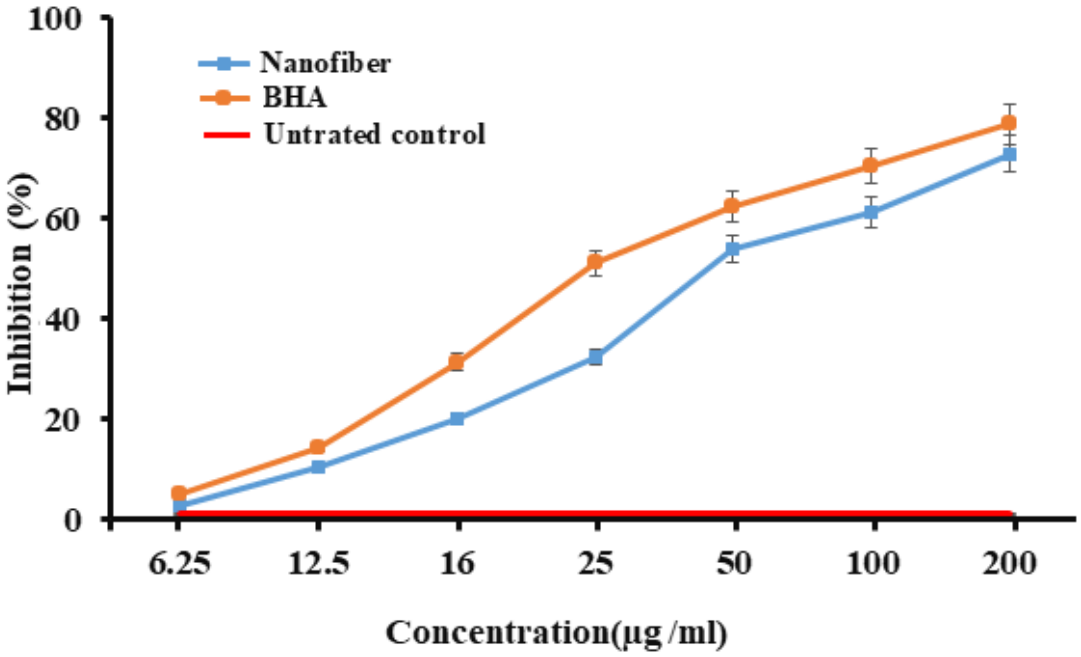


Figure 5. Scavenging effects of PLA/chitosan nanofibers on DPPH free radicals compared to BHA as a standard control (Mean ± SD, n=3)

3.2. Toxicity assey

The cytotoxicity of nanofibers containing various concentrations of amphotericin B were investigated on macrophages using flow cytometry assay. Significant differences were found between negative control and nanofibrous mats groups (p-value ≤ 0.001). The average value of CC_{50} for macrophages was about 14.21 µg/ml. Flow cytometry test results showed no cytotoxicity on macrophages. The international selectivity index (SI) in Eq. 1 was estimated at about 11.01 for nanofibers (Table 2).

$$SI\ (selectivity\ index) = CC_{50}\ (macrophages) / IC_{50}\ (promastigotes) \geq 10\ not\ toxic \quad \text{Eq. 1}$$

The viability of the nanofibrous mats with 1.25, 2.5 and 5 µg/ml of amphotericin B in comparision to untreated cells (92.04%) are 88.74%, 89.01%, and 69.43% (Figure 6). Therefore, it can be concluded that PLA/chitosan nanofibers as a novel nanostructure have low toxicity to macrophages for treat *leishmania* lesions.

Table2. Toxicity of Polylactic acid/chitosan nanofibers decorated with amphotericin B on macrophages and the lethal effect on *Leishmania* major promastigotes.

Compound	IC_{50} promastigotes (μg/ml)	CC_{50} macrophages (μg/ml)	SI
Amphotericin B loaded PLA/chitosan nanofibers	1.29	14.21	11.01

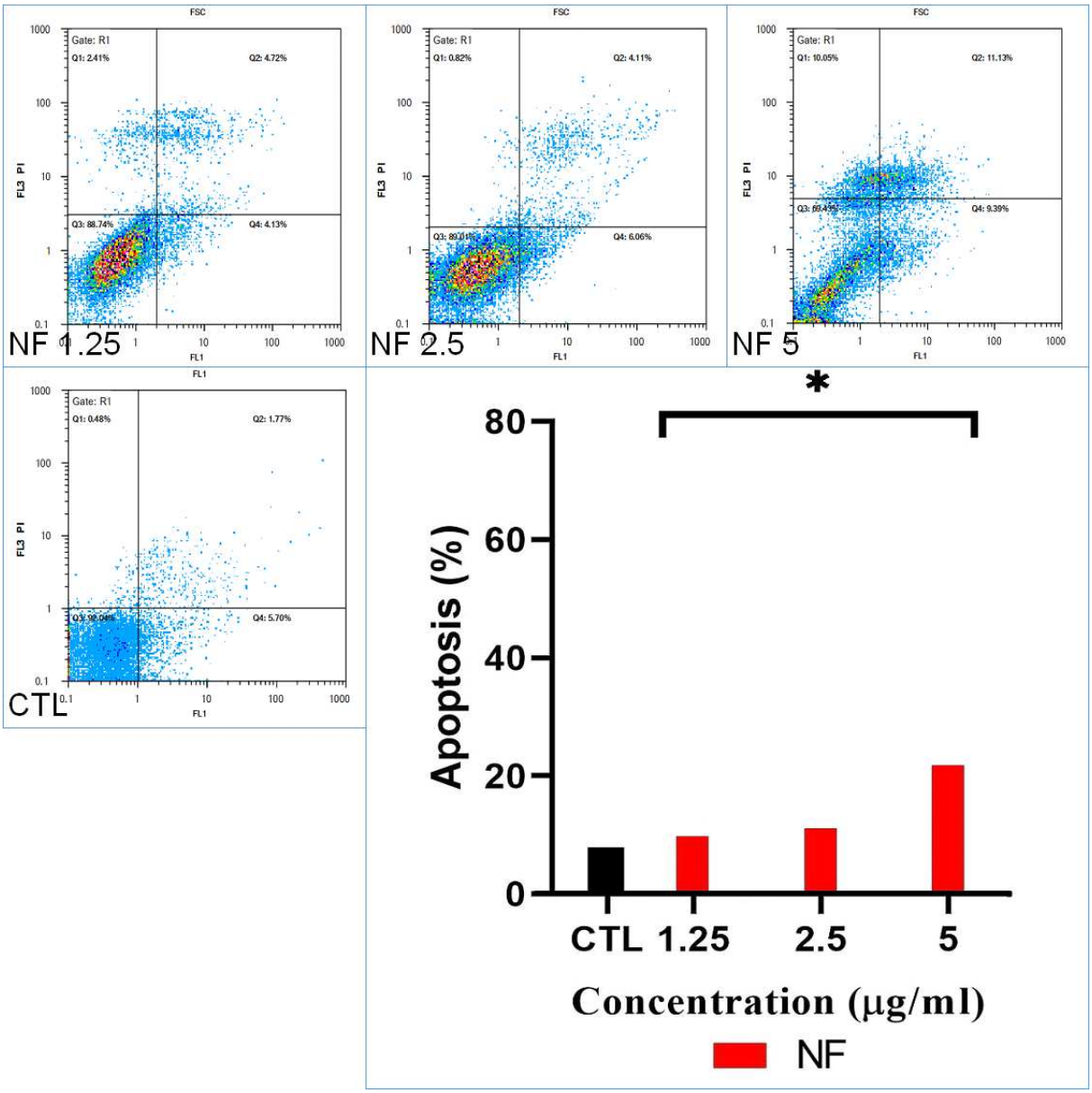
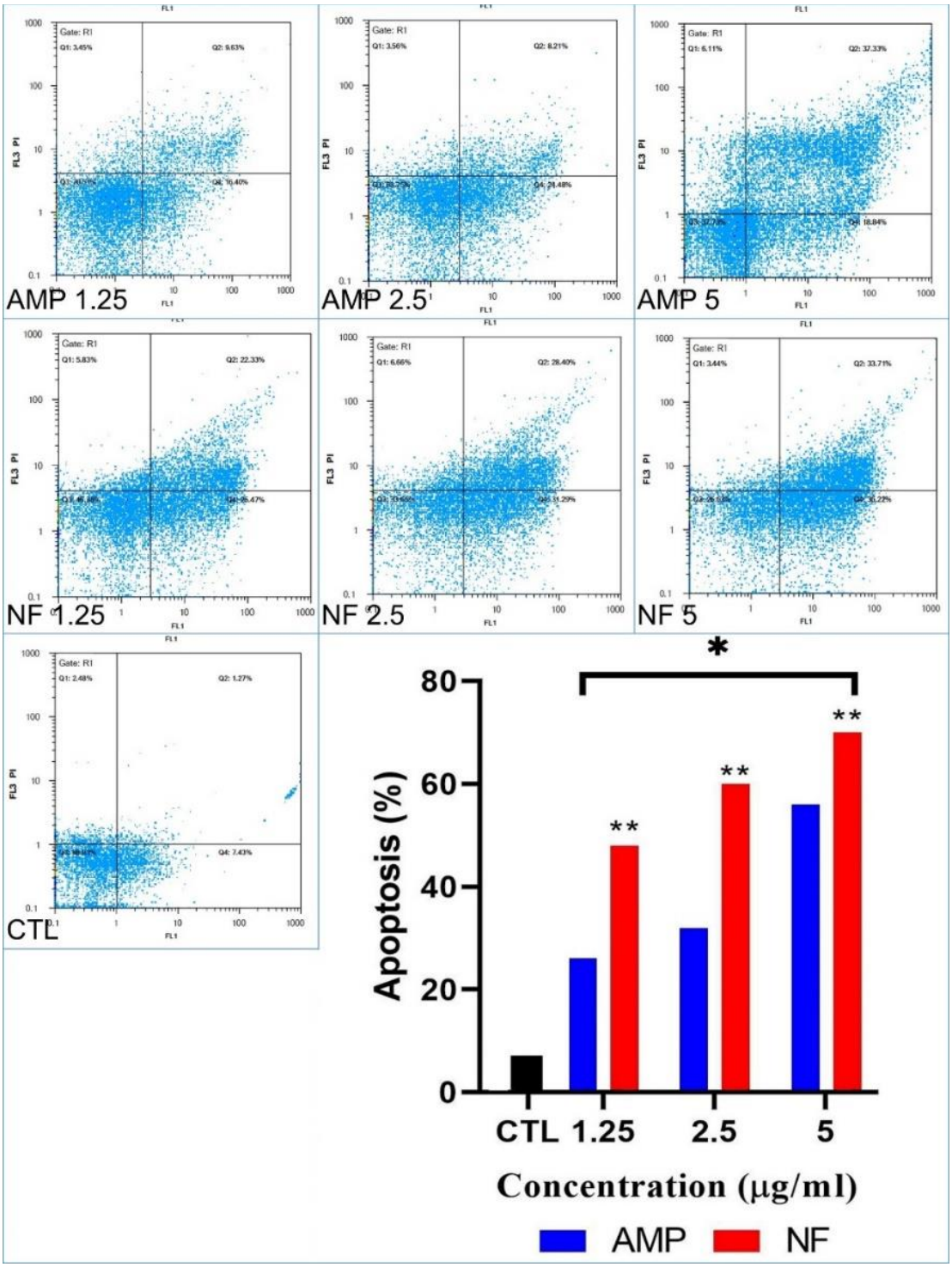


Figure 6. The percentage of the apoptosis macrophages at different concentrations of amphotericin B loaded nanofibers such as 1.25 µg/ml, 2.5 µg/ml and 5 µg/ml with control sample (Mean ± SD, n=2)

3.3. Assessment of parasitic apoptosis

According to the main purpose of this study to synthesize amphotericin B loaded nanofibers for treating cutaneous *Leishmaniasis*, the efficiency of these fibers towards the parasites was investigated. The *anti-Leschmaniasis* activities of different concentrations of amphotericin B

loaded nanofibers by flow cytometry method were shown in Fig. below. Drug-loaded nanofibers exhibited *anti-leishmaniasis* activity in a dose-dependent manner and killed 69.93%, 59.69%, and 47.80% of parasites by programmed cell death, respectively with 5, 2.5, and 1.25 µg/ml of amphotericin B loaded nanofibers, while in similar concentrations, conventional form of amphotericin B killed 56.17%, 32.69%, and 26.03% of parasites, respectively (Figure 7). The flow cytometry results (cell death) between controls and the treated cells were analyzed by analysis of variances (ANOVA). The average concentration of the amphotericin B loaded in PLA/chitosan nanofibers and conventional form of amphotericin B that prevented the growth of 50% of the promastigotes of *L.major* was 1.29 and 4.34 µg/ml, respectively. Significant differences were found between negative control and positive control and nanofibrous mats groups (p-value $\leq$ 0.0001).



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299 **Figure 7.** The percentage of the apoptosis of *Leishmania major* in different concentrations such
300 as 1.25 µg/ml, 2.5 µg/ml and 5 µg/ml with control sample (Mean ± SD, n=2) of Amp B loaded
301 nanofibers and conventional form of amphotericin B.

302

4. Conclusion

In this study, for the first time Polylactic acid/chitosan nanofibers were decorated with amphotericin B for in vitro treatment of *leishmaniasis*. The rate of drug release of PLA/ chitosan nanofibers structures after 400 min was 84%. Statistical analysis of the BHA showed a difference with PLA/chitosan nanofibers, although the antioxidant activity of nanofibrous mats were within acceptable range. Moreover, the 5 µg/ml of these drug loaded nanofibrous mats exhibited suitable *anti leishmanial* activity (killing 69.93% of parasites), which was comparable with conventional form of 5 µg/ml amphotericin B (killing 56.17% of parasites). The concentration of the nanofibers that prevented the growth of 50% of macrophages for PLA/chitosan nanofibers were 14.21 µg/ml. Due to the high toxicity of *anti leishmaniasis* drugs, the toxicity of proposed nanofibers evaluated as the SI parameter.

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Declaration of Competing Interest

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

Compliance with ethics requirements

This article does not contain any studies with human subjects.

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