

# Anti-Leishmanial Effects of Chitosan-Polyethylene Oxide Nanofibers Containing Berberine: An Applied Model for Leishmania Wound Dressing

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## What's Known

- Treatment of cutaneous leishmaniasis with conventional drugs is followed by numerous side effects. Hence, new treatments with natural compounds having minimum side effects deemed necessary.
- The use of electrospun nanofibers for wound dressing has opened a new horizon to researchers in regenerative medicine.

## What's New

- For the first time, a natural, sustainable, and non-toxic wound dressing with appropriate drug release and anti-leishmanial properties was produced based on chitosan.
- Various analyses indicated potent effects of the newly produced nano-scaffolds on *Leishmania major*, which can be used as a wound dressing to enhance the amelioration rate of the cutaneous lesions caused by this parasite.

## Abstract

**Background:** Leishmaniasis is the most important parasitic disease in Iran and is the third highest rate of rural cutaneous leishmaniasis in the world. Chitosan-polyethylene oxide nanocomposite fibers can be a suitable replacement for ordinary bandages. For the first time, in the absence of any published reports, the present *in vitro* study aimed to evaluate the anti-leishmanial effects of chitosan (CS)-polyethylene oxide (PEO)-berberine nanofibers on *Leishmania major*.

**Methods:** The present experimental study was conducted in 2018 in Tehran, Iran. The CS-PEO nanofibers containing berberine, as a natural anti-parasitic agent, were prepared using the electrospinning technique. Biocompatibility and fibroblast proliferation on nanofibers were investigated. In addition, the anti-leishmanial activity of CS-PEO nanofibers in both the promastigote and amastigote stages of *Leishmania major* was evaluated after parasite vital staining and MTT assay and compared to a control group. Statistical analysis was performed using SPSS software (version 18.0). Statistically significant differences were determined using the one-way ANOVA. The Duncan and Dunnett *post hoc* tests were used for within-group comparisons.  $P < 0.05$  was considered statistically significant.

**Results:** The results showed that nanofiber scaffolds with a mean diameter of  $77.5 \pm 19.5$  nm were perfect, regular, bead-free, and non-toxic, on which fibroblast cells grew well and proliferated. In addition, the optical density indicated that berberine 20% (w/v) significantly prevented promastigotes growth ( $IC_{50} = 0.24$   $\mu\text{g/mL}$ ) and amastigotes death ( $IC_{50} = 0.91$   $\mu\text{g/mL}$ ) compared with other concentrations and the control group.

**Conclusion:** The study on the cytotoxic effects showed that CS-PEO-berberine nanofibers had strong lethal effects on *Leishmania major* in promastigote and amastigote stages *in vitro*. Further studies are required to investigate the effects of this nanofiber on leishmanial ulcers in laboratory animals and clinical cases.

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**Keywords** • Biocompatible materials • Drug carriers • *Leishmania major* • Nanocomposites

## Introduction

The rural type of cutaneous leishmaniasis, caused by *Leishmania major*, is highly prevalent in Iran with 27,000 reported cases across 17 (55%) provinces. This parasitic disease is one of the

most common endemic diseases and has been the main focus of the Iranian national action plan for the prevention and control of diseases. Development of new drugs to treat leishmaniasis is still required and ongoing; however, drug therapy involves many side effects such as the risk of drug resistance in endemic countries.<sup>1-3</sup>

Tissue engineering is a potential alternative to treat a disease through the restoration and regeneration of the damaged tissues. In tissue manufacturing, an appropriate scaffold with physical and mechanical structures is required to provide adhesion of cells, growth, proliferation, migration, penetration of cells to nano-scaffold, specific morphology, and to create new tissue to replace the impaired body tissue.<sup>4</sup> In addition, researchers have focused on the use of nano-based drugs which are more suitable for their controlled drug-releasing properties. Nanofibers are a new type of materials used in medical applications. It has biocompatible and biodegradable compounds capable of stimulating immune systems, increasing the secretion of cytokines, and stimulating the cytotoxic T cells. Chitosan (CS) is a natural polymer, however, it cannot produce nanofibers alone due to its high viscosity. The reduced viscosity, after adding polyethylene oxide (PEO), can be due to the change in intra- and inter-molecular interactions of CS chains. PEO molecules bind onto the CS backbone, which leads to increased solubility of CS and reduced viscosity of the solution.<sup>5</sup> Considering the structural similarity of CS to the extracellular matrix, it is an excellent nano-scaffold for tissue regeneration. This substance is not produced by the human body and its presence stimulates the immune system and is recognized by receptors such as TLR-2, Dectin-1, and mannose.<sup>6,7</sup>

*Berberis vulgaris*, particularly its main component berberine, is a source of isoquinoline alkaloid. Its main ingredients, natural and crystalline substances have shown high potential in eradicating various parasites and *Leishmania* strains. Some studies have shown that berberine exhibits anti-leishmanial effects by regulating the mechanism of *mitogen-activated protein kinase* (MAPK) pathway in macrophages. Additionally, the anti-inflammatory effects of berberine *in vitro* have been proven in experimental studies on animals.<sup>8-10</sup> Electrospun nanofibers can significantly meet tissue regeneration requirements, such as gas exchange (oxygen), wound protection against infection, and prevention of dehydration. An ideal wound dressing should have high porosity and form an excellent barrier against micro-organisms. Moreover, it should easily detach from the tissue without causing any damage, absorb wound

secretions, prevent surface infection, and provide a good gas exchange between the wound and the environment. In this regard, various synthetic and natural polymeric dressings have been used in the quest for the ideal dressing.<sup>11</sup>

For the first time, in the absence of any published reports, the present *in vitro* study aimed to evaluate the anti-leishmanial effects of CS, PEO, and berberine nanofibers.

## Materials and Methods

### Chemicals and Instruments

The materials used in the present study were a medium molecular weight of CS and PEO (900 kD MW), berberine chloride hydrate (berberine), glutaraldehyde solution 50% (v/v), dialysis bags (12 kD), and [3-(4.5-dimethylthiazol-2-yl)-2.5-diphenyl tetrazolium bromide] (MTT) powder; all purchased from Sigma-Aldrich (USA). In addition, *Leishmania major* (MRHO/IR/75/ER) was obtained from Tehran University of Medical Sciences (Tehran, Iran).

The instrumentations were an electrospinning instrument (Lab2 ESI-II; Nano Azma, Iran), scanning electron microscope [SEM] (AIS2100; Seron Technology, Korea), transmission electron microscope [TEM] (Zeiss EM900; Carl Zeiss AG, Germany), atomic force microscope [AFM] (NanoWizard-II; JPK Instruments, Germany), FT-IR (Nexus 670; Thermo Nicolet, USA), and UV-Vis spectrometer (Analytik Jena AG, Germany).

### Nanofiber Preparation using Electrospinning

The CS-PEO mixture was prepared by slowly adding 0.27 g CS and 0.04 g PEO to an appropriate volume of 50% acetic acid. The prepared solution was stirred on a magnetic stirrer (300 rpm, 12-24 hours, 37 °C).<sup>12</sup> Berberine 0.5-50% (w/v) was slowly added to the CS-PEO solution and stirred well (3 hours at 37 °C) to produce a yellowish-homogeneous solution prior to electrospinning. A 5 mL syringe (needle size: 25 G) was filled with the polymeric solutions and electrospinning was performed at ambient temperature (feed rate: 0.1 mL/h, voltage: 16.5 kV, tip-to-target distance: 15 cm, and rotating speed: 400 rpm). The samples were then dried for 24 hours at 24 °C.

### Scanning Process and Image Formation

Electrospun nanofibers were sputter-coated with gold (Au) and SEM images were prepared. Unlike AFM shooting, which does not need to be prepared, the prepared sample for TEM is so thin that it is possible to cross the electron. Initially, a groove on aluminum foil was made and a grid size 200 mesh (Agar Scientific) was

placed inside it. The construction was then subjected to electrospinning for 3-5 minutes.<sup>13</sup>

#### *Cross-Linking of Nanofibers*

The electrospun nanofibers were placed on top of the 25% aqueous glutaraldehyde in a desiccator (Deltalab, Spain) under vacuum conditions for 24 hours at 24 °C. The nanofibers were then dried for 24 hours at 24 °C to remove the excess amounts of glutaraldehyde.<sup>14, 15</sup>

#### *Stability of Nanofibers*

To simulate human skin, 25 mg (7×7 cm<sup>2</sup>) of non-crosslinked and cross-linked nanofibers were weighed and immersed in *phosphate-buffered saline* (PBS), pH 5.5, for 24 hours at 37 °C. The morphology of the nanofiber structures was then studied using SEM.<sup>16</sup>

#### *Drug Release Rate and Kinetic Investigation of CS-PEO-Berberine Nanofibers*

Maximum absorption of berberine was calculated by UV-Vis spectrometry ( $\lambda_{\text{max}}=229$  nm). In accordance with the Beer-Lambert law, the calibration curve was drawn at 229 nm. Then, 25 mg of CS-PEO-berberine 20% was weighed and stored in a 12 kD dialysis bag with 5 mL of PBS (pH 5.5) at 37 °C. This was placed in a beaker containing 50 mL of PBS (pH 5.5) and subsequently positioned on a shaker (KBLee, Korea) at 37 °C. At different intervals of up to 2 weeks, 2 mL of the buffer was removed for analysis and replaced by the same amount of fresh buffer. The UV-Vis spectrometer was used to measure the amount of released drug.<sup>16, 17</sup>

#### *Evaluation of Nanofibers Porosity ( $\epsilon$ )*

Nano-scaffolds were first immersed in a graduated cylinder containing a known volume ( $V_1$ ) of 96% ethanol for 24 hours. The total volume of ethanol with nano-scaffolds was then noted as  $V_2$ . Subsequently, the volume difference ( $V_1-V_2$ ) was reported as  $V_3$ .<sup>18</sup> The porosity of the nano-scaffolds was then calculated according to the formula below.

$$\epsilon = [(V_1 - V_3) / (V_2 - V_3)] \times 100$$

#### *Biocompatibility Test and Evaluation of Cell Growth and Proliferation on Nanofibers*

Human fibroblast cells were applied to determine the biocompatibility of the nanofibers in accordance with a previous study (guidelines for maintaining cultured cells).<sup>19</sup>

#### *Investigation of Cell Viability on Nano-Scaffolds by MTT Assay*

At first, the nanofibers were sterilized with UV light (each side at an appropriate distance for 15

minutes). Dulbecco's Modified Eagle's medium (DMEM) complete medium (130  $\mu$ L) added with 50  $\mu$ L containing 10<sup>4</sup> fibroblasts was poured in a 96-well plate (24 hours, 37°C, 5% CO<sub>2</sub>) and the nanofibers containing 0.5-50% berberine were placed on them. Then, 20  $\mu$ L MTT was added and incubated for 4 hours, after which 200  $\mu$ L Dimethyl sulfoxide (DMSO) was added. After shaking, the optical absorption of the sample was read at 570 and 630 nm using a multi-mode reader (BioTek, USA).<sup>19, 20</sup>

#### *Evaluation of CS-PEO-Berberine Nano-Scaffold Inhibitory Effects*

To evaluate the inhibitory effects of CS-PEO-berberine nano-scaffolds against the promastigotes stage, the nanofibers were placed into the wells of 96-well bottom plates and added with 10<sup>4</sup> promastigotes/100  $\mu$ L followed by 24 hours incubation. Then, 10  $\mu$ L MTT was added to each well and incubated for 4 hours, after which it was centrifuged at 1000-2000 rpm. After adding 100  $\mu$ L DMSO, the absorbance was recorded.<sup>19, 20</sup>

#### *In Vitro Cultivation of Axenic Amastigote*

Fetal bovine serum (FBS) 20% (Gibco, USA) and 1% penicillin/streptomycin (Sigma-Aldrich, Germany) were added to RPMI-1640 and its pH was adjusted to 4.5. After adding 10<sup>6</sup> promastigotes, it was stored in 25 cm<sup>2</sup> flasks (Sigma-Aldrich, USA) in a humidified incubator (Mettler, Germany). The medium was then centrifuged (1800-2000 rpm) with warm PBS (three times) and incubated for 3-5 minutes at 37 °C. The process of transforming from promastigotes to amastigotes began on day 4 and reached its peak on day 6.<sup>21, 22</sup> The inhibitory effects of CS-PEO-berberine nano-scaffolds on the axenic amastigote stage were also evaluated. The process was in the same manner as the previous stage, except for using a suspension containing 10<sup>4</sup> axenic amastigotes.<sup>22</sup>

#### *Calculation of Minimum Inhibitory Concentration (MIC)*

Nanofibers were placed in each well of 96-well plates and 10<sup>4</sup> promastigotes per well were added, followed by an incubation period (72 hours, 37 °C, 5% CO<sub>2</sub>). The number of alive promastigotes was counted under a microscope and compared to the control group at 24, 48, and 72 hours, respectively. Subsequently, the MIC values were calculated according to the below formula.<sup>23, 24</sup>

MIC = [(mean number of live parasites in the control group - mean number of live parasites in the case group) / mean number of live parasites in the control group] × 100

### Statistical Analysis

All experiments were done in triplicate. The  $IC_{50}$  values and selectivity index were calculated using GraphPad Prism software (version 6.07). In addition, statistical analysis was performed using SPSS software (version 18.0). Statistically significant differences were determined using the one-way ANOVA. The Duncan and Dunnett *post hoc* tests were used for within-group comparisons.  $P < 0.05$  was considered statistically significant.

### Ethical Approval

The study was approved by the Ethics Committee of Shahid Beheshti University of Medical Sciences, Tehran, Iran (IR.SBMU.MSP.REC.1397.437).

## Results

### Evaluation of CS-PEO Nanofibers (90:10) with and without Berberine

Based on SEM images, the obtained nanofibers were regular and without beads. Their mean diameter was calculated using ImageJ software (figures 1A, 1B). The perfect bead-free and cylindrical nanofibers were obtained using the 0.5-50% (w/v) concentration. The mean diameter of the nanofibers was  $77.5 \pm 19.5$  nm in CS-PEO, which was enlarged by increasing the drug ratio (figures 1C-1F). In order to study the internal and 3D structure of nanofibers, the AFM and TEM images were used (figures 2A-2F).

### Analysis of Nanofibers Stability

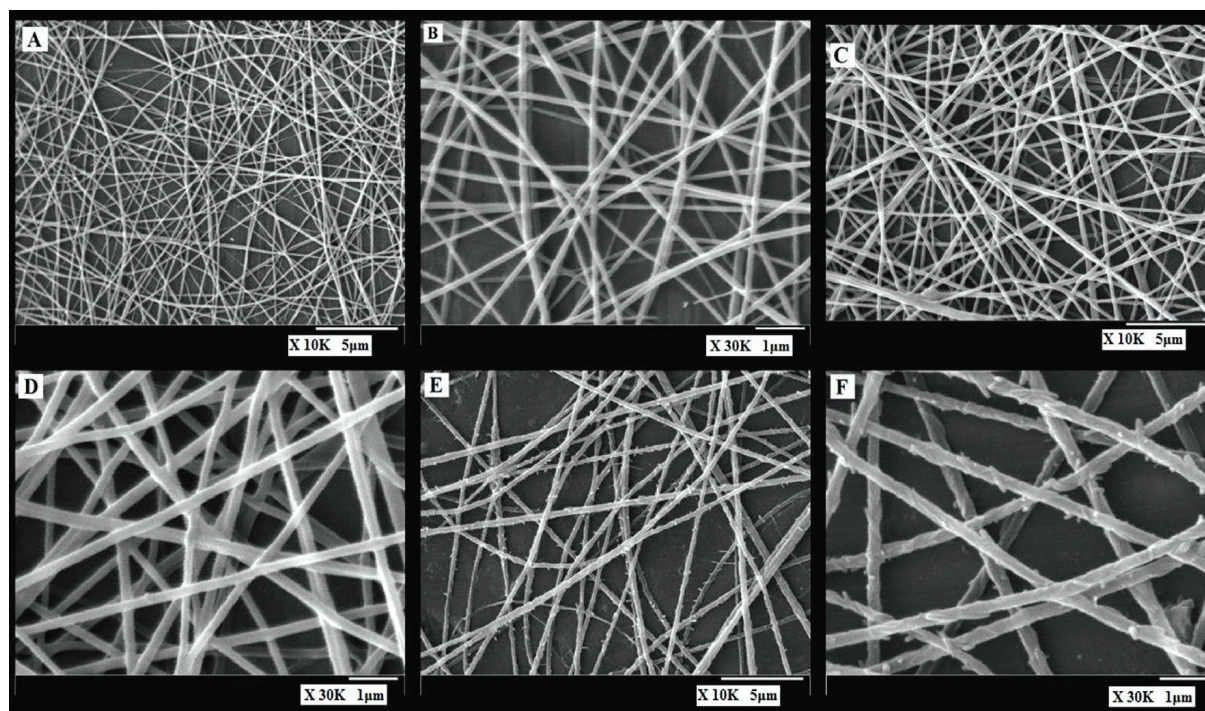
The non-crosslinked samples containing berberine, especially in high concentrations after immersion in PBS, lost their structure. This was related to CS nanofibers turning into film layers between the fibers. However, cross-linked samples with glutaraldehyde retained their structures (figures 2G-2J, 3A). The mean diameter of nanofibers increased after networking. The mean diameter of CS-PEO-berberine 50% nanofibers increased from  $159.4 \pm 30.7$  to  $202.3 \pm 27.4$  nm in an optimal state (figures 2I, 2J).

### Berberine Releasing Template of CS-PEO-Berberine Nano-Scaffolds

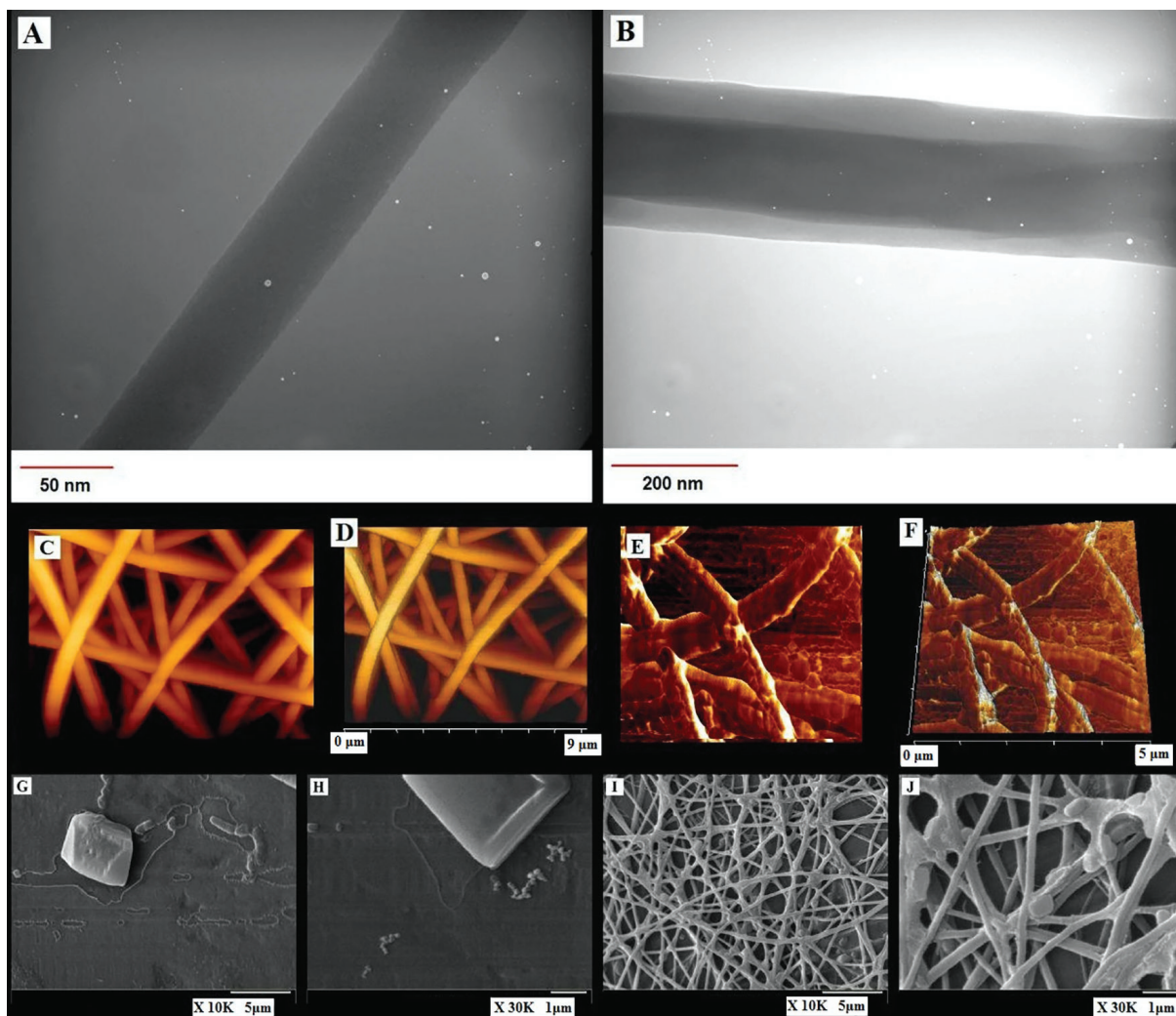
Figure 3B illustrates the berberine calibration curve ( $\lambda_{max} = 229$  nm). The drug release rate from nanofiber scaffolds after  $\approx 18$  hours was 50%. The rate in days 1, 2, and 3 was 64.75%, 75.4%, and 80%, respectively. The drug release continued up to day 14 (figure 3C).

### In Vitro Cellular Study

A suspension of fibroblast cells was equally mixed with trypan blue and examined using an optical microscope. The viability of the cells was more than 90% (figures 3D, 3E). In order to confirm biocompatibility, human fibroblasts were cultured on CS-PEO-berberine nano-scaffolds and their morphology was investigated using SEM (figures 3F, 3G).



**Figure 1:** Scanning electron microscope images illustrate electrospun chitosan-polyethylene oxide (90:10) nanofibers (A, B), electrospun chitosan-polyethylene oxide nanofibers containing berberine 0.5% (C, D), and berberine 4% (E, F) at different magnifications.



**Figure 2:** Transmission electron microscope images illustrate electrospun chitosan-polyethylene oxide-berberine 20% nanofibers (A, B). Atomic force microscope images of a 3D structure of prepared nanofibers are illustrated before (C, D) and after (E, F) berberine 20% loading. Stability study of chitosan-polyethylene oxide-berberine 50% non-crosslinked (G, H) and cross-linked nanofibers with glutaraldehyde (I, J) after 24 hours immersion in phosphate-buffered saline at 37 °C, pH 5.5.

#### Fourier-Transform Infrared Spectroscopy (FT-IR) Analysis

The FT-IR spectrum of CS-PEO nanofibers is shown in figure 4A. The broadband between 3,200-3,600  $\text{cm}^{-1}$  was attributed to the stretching mode of the O-H and N-H bonds in the CS and the O-H bond in the PEO backbone. The absorbent bands at 2,862 and 2,919  $\text{cm}^{-1}$  were attributed to the C-H symmetrical and asymmetrical stretching vibration of the  $\text{CH}_2$  group of CS, respectively. The 1,640  $\text{cm}^{-1}$  band showed stretching of the C=O of  $\text{CH}_3\text{CONH-}$  group of CS. These results indicated that hydrogen bonding occurred between CS and PEO molecules.

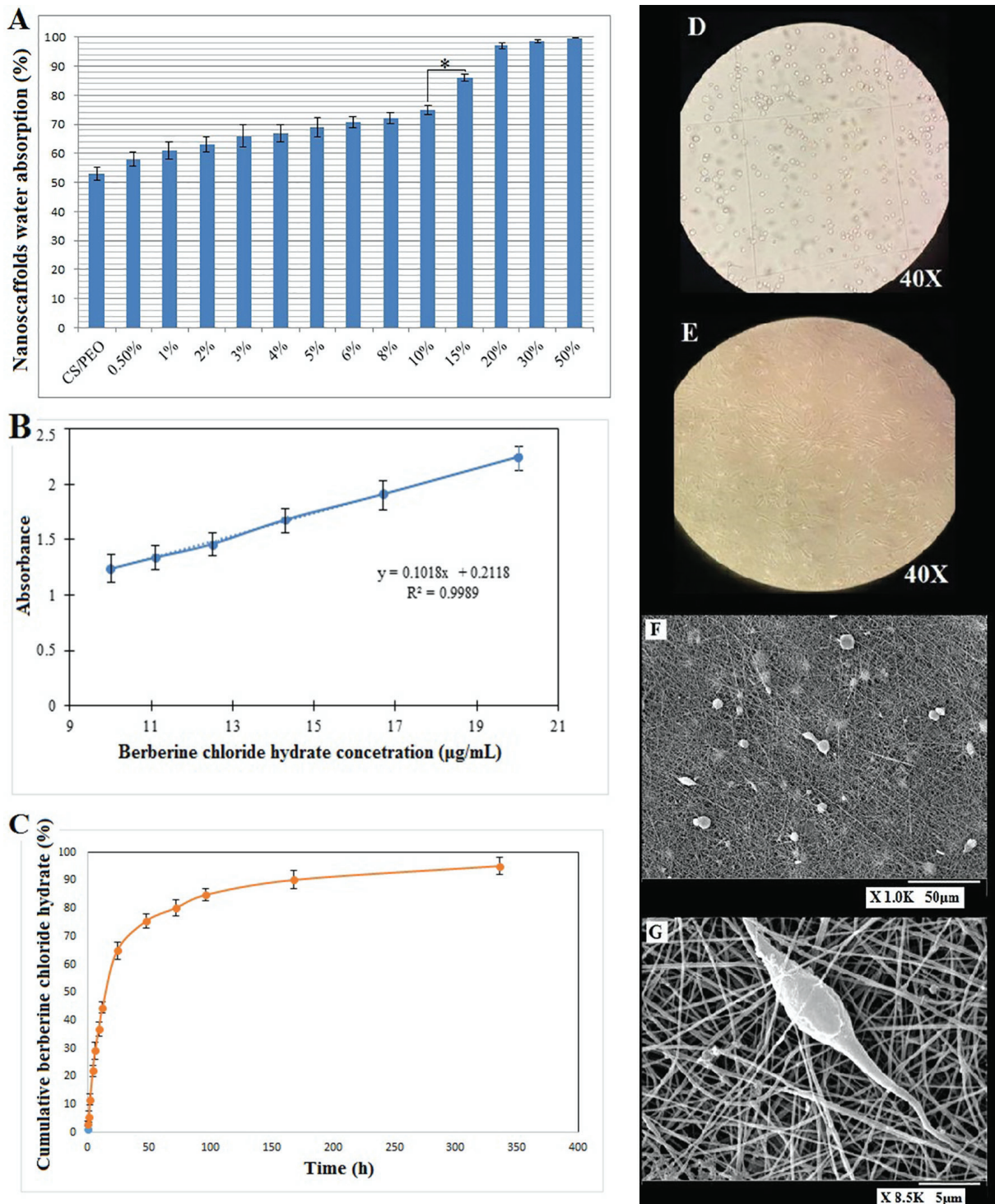
#### CS-PEO-Berberine Nanofibers

Aromatic vibrations appeared between 1,510 and 1,600  $\text{cm}^{-1}$ , 1,200 and 1,250  $\text{cm}^{-1}$  (in-plane CH bending and semicircle ring stretching) and at 1,100  $\text{cm}^{-1}$  (CH in-plane bending). The most

intense vibrations in the infrared spectrum belonged to the OCH groups, with the CH scissoring of the  $\text{CH}_2$  group in the dioxolane-type. The intense band at  $\approx 1,065 \text{ cm}^{-1}$  probably corresponds to the symmetric OCO stretch of the dioxolane-type ring (figure 4B). The FT-IR spectrum of berberine revealed the existence of a methoxyl group (peak at 2,917.55  $\text{cm}^{-1}$ ). The peak at 1,634.08  $\text{cm}^{-1}$  is believed to correspond to the iminium ( $\text{C}=\text{N}^+$ ) double bond present in the molecule. The broad strong peak at 3,411.31  $\text{cm}^{-1}$  (figure 4C) corresponds to O-H stretching of adsorbed water. Moreover, the signals at 1,598.83  $\text{cm}^{-1}$  represent the aromatic C=C bending.

#### CS-PEO-Berberine Effect on Human Fibroblast Cells

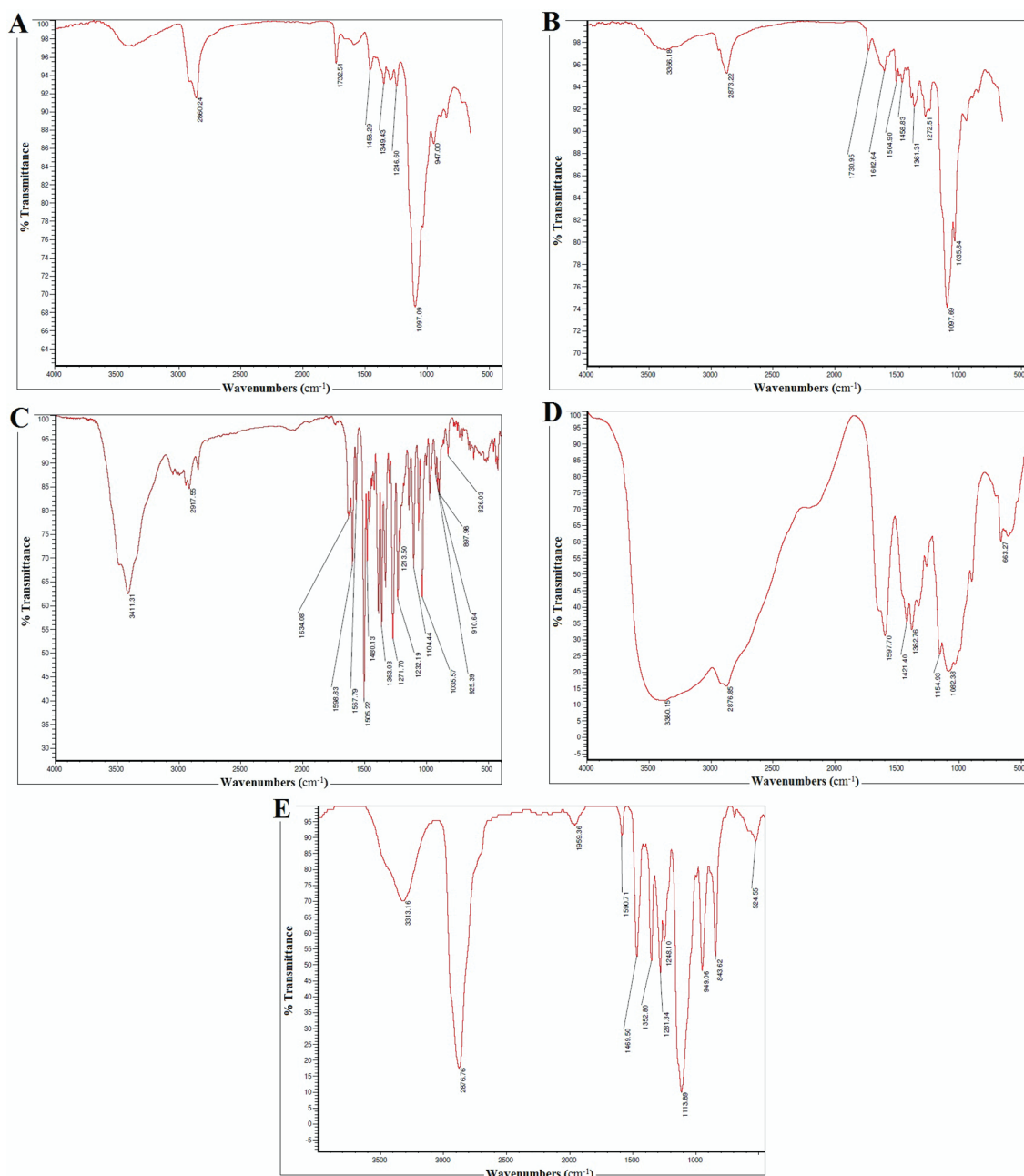
The CS-PEO-berberine 30% and 50% nano-scaffolds had a strong cytotoxic effect on human fibroblast cells and were excluded from the study.



**Figure 3:** The images display A) the swelling diagram of chitosan-polyethylene oxide nanofibers with and without berberine in phosphate-buffered saline, pH 5.5 at 37 °C; B) calibration curve of berberine chloride hydrate; C) the release percentage of berberine from electrospun mats containing berberine 20% based on time; D) optical, and E) inverted microscopic images of fibroblast cells cultured in Dulbecco's Modified Eagle's medium containing 10% FBS on day 6; F, G) Scanning electron microscope images of fibroblast cells on chitosan-polyethylene oxide-berberine 20% nanofibers at different magnifications.

Except for these, the rest of the nano-scaffolds, including CS-PEO alone and other berberine concentrations had a moderate toxic effect on fibroblast cells and were not dose-dependent. However, berberine alone significantly inhibited the growth of these cells in all concentrations in

both a dose- and time-dependent manner. On the other hand, the lethal effects of different doses of berberine showed a significant difference in comparison with the control group ( $P=0.001$ ), which were higher at 15% and 20% doses than at other doses (tables 1 and 2).



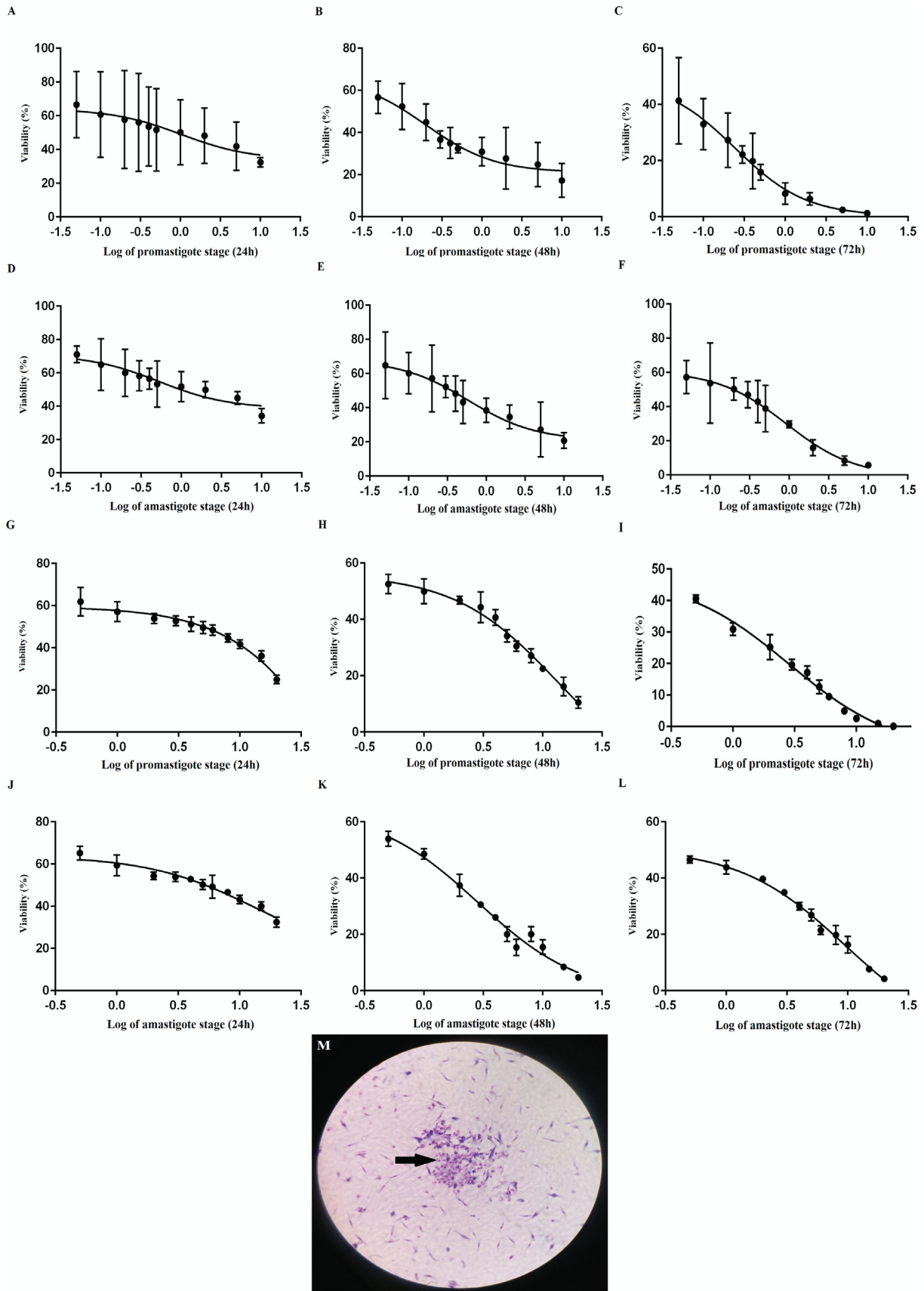
**Figure 4:** The Fourier-transform infrared spectroscopy spectrums show A) chitosan-polyethylene oxide nanofibers, B) chitosan-polyethylene oxide-berberine 20% nanofibers, C) berberine, D) pure chitosan, and E) pure polyethylene oxide.

#### Evaluation of CS-PEO-Berberine Inhibitory Effect on Promastigote and Amastigote Stages

The CS-PEO nano-scaffolds, with and without berberine, inhibited the *Leishmania major* promastigote stage in a specific. The half maximal inhibitory concentration (IC<sub>50</sub>), which significantly depended on the berberine percentage. The anti-amastigote effects of the aforementioned nano-scaffolds were similar to a specific IC<sub>50</sub> like promastigote stage.

There was a significant difference between the cytotoxic effect of CS-PEO-berberine in doses <1% versus >1% on parasite (P=0.04). Berberine alone was dose-dependent for lethality

in both the promastigote and amastigote stages. CS-PEO with berberine could significantly prevent parasites growth in a dose-dependent manner. As shown in figure 5, the inhibition reached the highest rate in CS-PEO-berberine 20% (P=0.001). Among the three cell types, the highest cytotoxic effect was observed in *Leishmania major* promastigote and there was a significant difference between the effect of berberine on fibroblast and amastigote than on promastigote. The nano-scaffolds containing ≤4% berberine had significantly lower cytotoxic effects on the parasites (P=0.001). In addition, MTT results demonstrated that the mean



**Figure 5:** The effect of berberine chloride hydrate in different concentrations on A-C) *Leishmania major* promastigote and D-F) amastigote stages; G-I) chitosan-polyethylene oxide nano-scaffolds containing different percentages of berberine effect on *Leishmania major* promastigote and J-L) amastigote stages; M) the degenerative changes of *Leishmania major* promastigotes after the chitosan-polyethylene oxide-berberine 20% nanofibers treatment 100 $\times$ .



**Table 1:** Berberine chloride hydrate IC<sub>50</sub> values with respect to the growth rate of human fibroblast cells, promastigote, and amastigote stages of *Leishmania major*

Time (hours)	IC <sub>50</sub> values (µg/mL)			P value*
	Fibroblast	Promastigote	Amastigote	
24	6.9±0.88	1.023±0.061	0.53±0.055	0.001
48	3.4±0.17	0.197±0.018	0.58±0.042	
72	3.5±0.22	0.24±0.019	0.91±0.034	

All IC<sub>50</sub> were significant for all berberine concentrations in both dose- and time-dependent manner; Data are expressed as mean±SD (n=3), \*One-way ANOVA tests, P<0.05 was considered significant

**Table 2:** Berberine chloride hydrate selectivity index with respect to the *Leishmania major* promastigote and amastigote stages

Time (hours)	Selectivity index (SI)		P value*
	Promastigote	Amastigote	
24	6.74	13.01	0.001
48	17.25	5.86	
72	14.58	3.84	

\*One-way ANOVA tests, \*P<0.05 was considered significant

**Table 3:** Evaluation of the effect of various nano-scaffolds on *Leishmania major* in the promastigote stage.

Variable	MIC (%)			P value*
	24h	48h	72h	
CS-PEO	40.7±2.53	55.3±2.15	62.7±4.6	0.001
CS-PEO-berberine 0.5%	48.3±2.08	60.2±2.32	66.0±3.4	0.001
CS-PEO-berberine 1%	54.9±3.5	65.0±1.98	71.1±2.13	0.04
CS-PEO-berberine 2%	59.0±2.13	67.6±2.82	73.9±3.12	0.001
CS-PEO-berberine 3%	66.2±3.81	71.5±4.6	76.2±3.08	0.001
CS-PEO-berberine 4%	76.0±5.23	82.2±3.54	88.0±1.88	0.001
CS-PEO-berberine 5%	78.9±2.87	83.0±3.2	90.0±4.07	0.001
CS-PEO-berberine 6%	84.6±2.23	91.0±1.2	98.1±1.17	0.001
CS-PEO-berberine 8%	95.7±0.9	99.1±0.7	100±0.0	0.001
CS-PEO-berberine 10%	97.5±0.93	100±0.0	100±0.0	0.001
CS-PEO-berberine 15%	100±0.0	100±0.0	100±0.0	0.001
CS-PEO-berberine 20%	100±0.0	100±0.0	100±0.0	0.001

\*One-way ANOVA tests, P<0.05 was considered significant; Data are expressed as mean±SD (n=3); CS-PEO: Chitosan-polyethylene oxide

optical absorption in the studied groups had a significant difference in various concentrations of the drug and had a significantly higher mean light absorption in the CS-PEO-berberine 0.5% (P=0.001). The MIC test demonstrated the lowest level of anti-leishmanial agents that inhibited parasite growth. The MIC results showed that an increase in drug concentration increased the inhibitory effect on promastigotes in all prepared nanofibers. As shown in table 3, this effect intensified as time increased (P=0.001).

## Discussion

The new nanofibers were found to be biocompatible, stable, and non-toxic. They had a high drug-release capacity, which could be a suitable alternative treatment considering their powerful anti-leishmanial effects. A study on electrospun nanofibers, with different percentages of CS and PEO showed that

CS-PEO (90:10) maintained their structure in water and had a better cell adhesion. The results were consistent with those of synthesized nanofibers and cell cultures.<sup>5, 25</sup>

In the present study, the swelling degree of nanofibers improved by increasing the amount of berberine, which increased the release of active material from the nanofiber structure. A higher swelling degree indicated that the structure of nanofibers contained a higher level of moisture. As a direct result, the wound surface remains moist during a treatment and prevents the nanofibers from sticking. In addition, optimal oxygen exchange through the dressing improves the wound healing process. These findings were in line with other studies on CS-PEO nanofibers containing green tea or *Lawsonia inermis* extract.<sup>12, 26</sup>

A previous study reported that water-soluble nano-scaffolds dissolved and destructed in aqueous and *in vivo* conditions after about 6

hours and turned into a jelly state. A cross-link between hydrophilic polymers was proposed as an appropriate method to prevent such degradations.<sup>27</sup> Glutaraldehyde can cross-link with CS due to active aldehyde groups. Additionally, it can improve nanofiber properties such as water absorption, stability, solubility, and reduce its biodegradability. The cross-linked CS nanofibers produced in the present study were stable, in the same conditions as the human skin, and retained their structure. In line with other studies,<sup>12, 24</sup> their use as *in vivo* wound dressing is recommended. The porosity analysis showed that the produced nano-scaffolds had a high porosity percentage ( $\approx 90\%$ ), which was higher than in a previous study.<sup>18</sup> It indicated the high quality of the produced nano-scaffolds for maintaining an optimal gas exchange (oxygen) between the wound and the environment. Note that in tissue engineering, a minimum of 80% porosity is required to achieve a uniform distribution of cells throughout the scaffolds.

In the present study, drug release from the nano-scaffolds occurred in four stages. First, the so-called “high burst release” at the beginning when a significant amount of the drug was released at high speed. Second, a sustained drug release whereby the release was based on the level of penetration. Third, the release of the drug trapped deep in the scaffolds. The final phase was a slow decrease of drug release and even polymer degradation in a shallow curve slope. The observed multi-phase process was in line with a previous study.<sup>24</sup> TEM images indicated that the core-shell structure in the nanofiber was well-formed as a thin-walled uniform tube. The AFM results also confirmed the 3D structure of the nano-scaffolds. SEM images of the fibroblasts showed a stretched morphology, width, length, size, and proper adhesion of these cells on the nano-scaffolds. In line with previous studies, the results confirmed good biocompatibility of the produced nanofibers.<sup>13, 19</sup>

We found that the fibroblastic cells were well grown on the nanofibers, which indicated the non-toxicity of the produced nano-scaffolds. This was also confirmed in another study that investigated the wound healing process using CS scaffolds. They reported that CS accelerated the wound healing process through increased activity of inflammatory cells, polymorphonuclear leukocytes, macrophages, and fibroblasts.<sup>28</sup> In another study, CS and PEO were used in nano-scaffolds (for wound dressing) to culture L<sub>929</sub> fibroblastic cells *in vitro*.<sup>29</sup> They reported increased proliferation of fibroblasts and concluded that the nano-scaffolds could be

used as a wound dressing. Their finding was in line with the results of our cell analysis of the produced nano-scaffolds. CS-PEO nano-scaffolds containing berberine did not have dose-dependent effects on fibroblast cells. Therefore, the MTT test was performed on different concentrations of berberine, which showed that berberine alone exerted a dose-dependent effect on fibroblast cells. The results were similar to that of a test on macrophage cells. In line with other studies,<sup>8, 9</sup> we believe that berberine is non-toxic in conventional clinical doses. It is also not cytotoxic nor mutagenic, but it has more side effects in higher doses. Although berberine revealed significantly high cytotoxicity in host cells, it is harmless to mammals in normal doses.

Our results on CS anti-parasitic activity, in line with a previous study,<sup>30</sup> showed that CS alone had anti-leishmanial properties. While our results indicated strong anti-leishmanial effects of berberine, another study reported that berberine exerted an anti-leishmanial effect by adjusting the pathway of MAP-kinase in macrophages.<sup>10</sup> The results of the MIC test indicated that a reduction in the number of promastigotes was significant in the medium of all nano-scaffolds containing berberine. Also, because of the influence of the nano-scaffolds, the parasite growth decreased with the increase of the drug percentage and time. Therefore, the nano-scaffolds containing 15% or 20% berberine completely eradicated the promastigotes on the first day. The reported cytotoxic and anti-leishmanial effects of berberine alone without loading on nano-scaffold were in line with the results of the present study.<sup>8, 9</sup> The anti-amastigote effects of the produced nano-scaffolds were dependent on a specific IC<sub>50</sub> similar to those in the promastigote stage. The effect was more prominent in the promastigote stage; similar to the results of the effect of berberine on *Leishmania* parasite.<sup>8, 9</sup> Furthermore, the CS-PEO-berberine 20% nano-scaffold was superior to other concentrations of berberine in reducing the number and complete eradication of *Leishmania in vitro*. Moreover, it caused the eradication of more parasites, which were affected by nano-scaffolds. The inhibitory effect of the produced nano-scaffolds on promastigotes was stronger, which could be related to structural, morphological, and biochemical differences. A similar finding was reported in other studies that investigated the effects of berberine alone on the amastigote and promastigote stages.<sup>8, 9</sup>

The main strength of the present study was combining the properties of CS and berberine as natural materials. It provided a suitable wound dressing with anti-parasitic properties

for the amelioration of wounds caused by *Leishmania major*. This achievement was in line with the main goal of the study, which was to make practical use of nanofibers. However, the effectiveness of the dressing on wound healing in humans needs to be confirmed. The main limitation of the study was the lack of testing on animal models to evaluate its medicinal effect.

### Conclusion

The newly produced nanofibers showed high stability and a good anti-leishmanial effect on both the promastigote and amastigote stages of *Leishmania major*. The high porosity of the produced nanofibers was indicative of its use as a suitable wound dressing. Further studies are required to investigate the effects of this nanofiber on leishmanial ulcers in laboratory animals and clinical cases.

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