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Impregnated Nanofibrous Mat with Nanogel of *Citrus sinensis* Essential Oil as a New Type of Dressing in Cutaneous Leishmaniasis

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Abstract: Leishmaniases are a group of diseases caused by the Leishmania genus. Essential oils (EO)s have recently received more attention for the development of new green drugs. In this study, *Citrus sinensis* EO was used as an antileishmanial agent; its half-maximal inhibitory concentration (IC_{50}) against promastigotes of *Leishmania tropica* and *Leishmania major* was observed at 151.13 and 108.31 µg/mL. After that, the nanoemulsion-based nanogel of *C. sinensis* was prepared to improve its stability, potency, and facilitated topical usage. By adding carbomer 940 (2% w/v) to the prepared nanoemulsion with a 225 ± 7 nm droplet size, the nanogel was prepared. The nanogel was then impregnated on the electrospun nanofibers of chitosan-polycaprolactone, diameter = ~ 200 nm. The prototype's leishmanicidal effect was substantially better than the non-formulated EO; both species' viabilities were reduced to ~ 0%. The prepared sample could be used as a new type of dressing for cutaneous leishmaniasis; moreover, it could be considered an excellent candidate for *in-vivo* studies.

Keywords: *Citrus sinensis*: *Leishmania tropica*; *Leishmania major*; chitosan-polycaprolactone nanofibers; nanogel.

1. Introduction

Obligate intra-macrophage protozoa of the *Leishmania*s genus are responsible for leishmaniases, a group of vector-borne diseases [1]. The three main forms of leishmaniasis are cutaneous leishmaniasis, visceral leishmaniasis, and mucocutaneous leishmaniasis; they are induced by around 20 different species *Leishmania* [2]. Around ~100 countries in tropical and subtropical regions are involved with cutaneous leishmaniasis, the most common form of leishmaniasis [3, 4]. *Leishmania major* and *Leishmania tropica* in the old world, including Iran, Afghanistan, Saudi Arabia, and Syria, are responsible for cutaneous leishmaniasis in rural and urban areas, respectively [5, 6].

Essential oils (EOs) are naturally oily liquids secreted as secondary metabolites in the aromatic plants. They are extracted using different approaches such as hydro-distillation, steam distillation, and dry-distillation from different parts of plants, including stem, bark, and fruit [7, 8]. EOs possess many biological activities, such as the leishmanicidal effect. For instance, the potency of *Citrus sinensis* essential oil (CSEO) against *L. panamensis* and *L. braziliensis* has been reported previously [9]. Recently, the volatility of EOs could be controlled by formulating them into different nanoformulations, including nanoemulsions, polymeric nanoparticles, nanogels, and niosomes [10, 11]. Nanogels are good candidates for topical/transdermal applications; they possess many unique behaviors like fluidic nature, acquiring any shape during penetration, and modulating skin barrier for better penetration [12, 13].

Electrospinning is a versatile and straightforward technique for preparing nanofibers (NFs) [14, 15]. Electrospun NFs have been widely used in medical and health applications; for example, the use of NFs of chitosan (Chi) and polycaprolactone (PCL) in tissue engineering, wound dressing, and filtration [16, 17]. Moreover, the encapsulation of active ingredients into electrospun NFs is common. However, some practical challenges have remained still; electrospinnability and co-dissolving cargo and polymers, especially when the cargo is volatile (e.g., EO) [18, 19]. Furthermore, for observing the effect of a drug, the concentration must be reached to a certain range, while EO/drugs' weight could be loaded in the NFs are low. Thus, after-treatment methods may provide a useful solution for these issues.

In this study, the leishmanicidal activity of CSEO against promastigotes of *L. major* and *L. tropica* were first investigated. After that, for stability and potency improvement, a nanoemulsion-based nanogel of CSEO was prepared. To easy use in a topical manner, the nanogel was then impregnated on the Chi-PCL electrospun NFs. Finally, the leishmanicidal effect of those was compared.

2. Materials and Methods

2.1. Materials.

CSEO was purchased from Green Plants of Life Co. (Iran). Tween 80, PCL (80000 Mw), low molecular Chi (75-85% DD), Sodium hydroxide (NaOH), Phosphate Buffered Saline (PBS) were bought from Merck Chemicals (Germany). Pasteur Institute of Iran supplied *L. major (MHOM/IR/75/ER)* and *L. tropica (MHOM/SU/74/K27)*. Hexafluoro-2-propanol (HFIP), as the solvent of polymers in electrospinning, was purchased by SUVCHEM Co. (India). Carbopol 940 was obtained from SDFCL Co. (India).

2.2. GC-MS analysis.

Ingredients of CSEO were identified using GC-MS analysis, as described in our previous report [20].

2.3. Preparation and characterization of Chi-PCL NFs.

2.3.1. Preparation.

Powder of Chi (1% w/v) and granules of PCL (14% w/v) was dissolved in HFIP, and were mixed 0.75-3.5% w/v (24 h, room temperature). The Chi-PCL solution was then loaded in a 10 mL syringe (internal diameter 12 mm) connected to a blunted metal needle in an

electrospinning machine (Fanavaran Nano-Meghyas Co. Iran). Also, a power cable (15 kV) was attached to the needle. The polymer solution was injected (0.8 mL/h) using a syringe pump, and the distance between the needle and the rotating collector (100 rpm) was fixed at 100 mm. The collector's surface was wrapped with aluminum foil to facilitate the separation of the prepared NFs [21]. Morphology, size, and characterization of the obtained fibers were investigated using scanning electron microscopy (SEM), water contact angle measurement, and Attenuated Total Reflection Fourier Transform Infrared (ATR-FTIR).

2.3.2. Scanning electron microscopy.

SEM was used to investigate the morphology, size, and size distribution of the NFs (TESCAN-Vega 3, Czech Republic). The samples were coated with gold vapors. An image processing software (Free version of Digimizer, MedCalc Software Ltd, Belgium) was used to analyze the diameter of NFs.

2.3.3. Water contact angle measurement.

The wettability of the NFs was investigated using a contact angle measurement instrument (Sharif Solar Co. Iran). Seven µL of de-ionized water was injected on the NFs mat (1 cm × 1 cm), and its contact angle was recorded at 5 s [22].

2.3.4. ATR-FTIR.

The preparation of Chi-PCL NFs was confirmed by identifying the functional groups of the used polymer and final NFs. The study was performed using ATR-FTIR analysis in the wavelength range of 3500-500 cm^{-1} (Bruker Company, Model Tensor II, USA).

2.4. Preparation and characterization of nanoemulsion-based nanogel.

2.4.1. Nanoemulsion.

Oil in water nanoemulsion of CSEO was prepared using the spontaneous method. The CSEO (35 µL) and tween 80 (125) were mixed at 2000 rpm for 10 minutes. Distilled water was then added drop-wise up to the desired volume (5000 µL) and stirred for 35 min. Droplet size and droplet size distribution (SPAN) of the nanoemulsion were evaluated using a DLS type apparatus (dynamic light scattering, scatteroscope, K-ONE NANO. LTD, Korea). SPAN was calculated by d90 – d10 / d50, where d is the diameter of the droplets and x (i.e., 10, 50, and 90) are percentile of droplets with smaller than the defined diameter.

2.4.2. Nanogel.

The low viscosity of nanoemulsions has challenged their topical application. Thus, by adding a thickening agent, carbomer 940 (2% w/v), the nanoemulsion was transformed into a nanogel. Carbomer was first hydrated in the nanoemulsion (120 rpm, overnight, ambient temperature). The sample's pH was then adjusted from 4 to 6.5-7 using an aqueous solution of NaOH (25% w/v) for completing the gelling process. After that, the viscosity of nanogel was investigated using a rheometer machine at 25 ºC (Anton Paar rheometer Company, model MCR-302, Austria). Besides, a blank gel was also prepared using the same manner; only CSEO was not used.

2.4.3. Impregnated nanogel on the surface of the NFs.

Circular pieces of the Chi-PCL NFs with a diameter of 10 mm were punched. The nanogel (9.15 ± 0.1 mg) was then impregnated on each piece's surface, named NFsGel. Furthermore, the blank gel was also impregnated on other pieces and named NFsGel(-oil).

2.5. Evaluation of the leishmanicidal activity of CSEO, NFsGel, and NFsGel(-oil).

Serial dilution of CSEO in the concentration range of 5120 - 20 µg/mL was prepared using PBS's aqueous solution (containing 0.05% v/v DMSO) as a solvent. The leishmanicidal activity of the serial dilution and NFsGel, and NFsGel(-oil) was investigated using MTT assay. 400 µL/well of the serial dilution or 400 µL/well of the PBS solution containing pieces of NFsGel or NFsGel(-oil) was first added to 48-well plates, separately. After that, 400 µL/well of the suspension of each promastigote of *L. major* and *L. tropica* at the logarithmic phase was added. Promastigotes were cultured in the RPMI complete medium (10% Fetal Bovine Serum and 1% penicillin-streptomycin); their number was set 250,000/well.

After incubating the treated plates (24 h at 25 ºC), 50 µL of MTT solution (0.5 mg/mL) was added to each well and incubated for another 4 h. Finally, 200 µL/well of DMSO was added, and absorbance (A) of wells was read at 570 nm. The viability at each well was calculated by equation 1. This test was performed in triplicates; three control and blank groups were considered in each replicate. Control groups were filled with 800 µL PBS solution, and blank groups contained 400 µL RPMI complete medium (without promastigotes) and 400 µL PBS.

Viability (%) = ((Mean A sample-Mean A blank)/(Mean A control-Mean A blank))×100 (1)

3. Results and Discussion

3.1. Ingredients of CSEO.

Overall, 32 components were identified in the CSEO using GC-MS analysis. Five major ingredients with high amounts are limonene (71.264%), *trans*-p-2,8-menthadien-1-ol (4.956%), *cis*-limonene oxide (2.587%), *trans*-limonene oxide, (2.294%), and *trans*-carveol (2.906%).

3.2. The leishmanicidal activity of CSEO.

The leishmanicidal activity of CSEO on promastigotes of *L. tropica* and *L. major* is demonstrated in Figure 1. Because both promastigotes' viability was ~ 95% at the concentrations of 10 - 40 µg/mL, these concentrations possess negligible effect. However, by increasing the concentration to 160 µg/mL or higher levels, the viabilities were reduced to ~ 10%. The only point that showed a moderate effect on the promastigotes was 80 µg/mL; the viability of *L. major* and *L. tropica* were approximately 30% and 25%, respectively. Therefore, this concentration was chosen to compare the leishmanicidal effect of CSEO and its nano formulated form.

Furthermore, IC_{50}s (lower and upper confidence limits) of *L. tropica* and *L. major* were observed at 151 (93 - 245) µg/mL and 108 (71 - 164) µg/mL, respectively (CalcuSyn free version, Biosoft, UK). The leishmanicidal effect of other EOs against these promastigotes has also been found in the literature. For instance, IC_{50}s of EOs of *Zataria multiflora* and *Cymbopogon citratus* against *L. tropica* was reported at 89.30 and 52.00 µg/mL, respectively

[23, 24]. Besides, EOs of *Cymbopogon citratus*, *Rosmarinus officinalis*, and *Satureja bakhtiarica* with IC_{50}s of 149.10, 260.00, and 150.00 µg/mL, respectively, also showed potency against *L. major* [25-27].

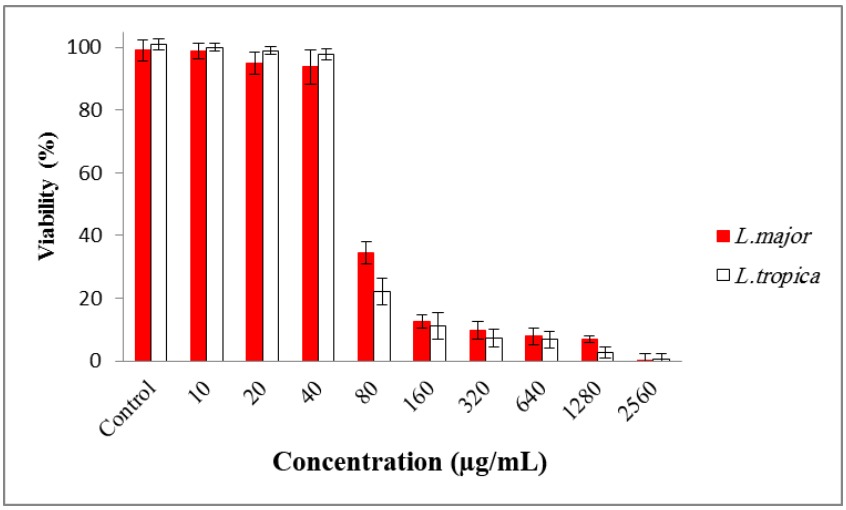

Figure 1. The leishmanicidal activity of CSEO.

3.3. Prepared nanofibers and their characteristics.

The SEM image of the prepared NFs with a 203 ± 29 nm diameter is shown in Figure 2A. As the water contact angle with the NFs mat's surface was 109º ± 2. It confirmed that the surface had a moderate hydrophobic property (Figure 2 B & C).

Chi is a cationic, biocompatible, and biodegradable biopolymer obtained by chitin's deacetylation [28, 29]. It posses many biological activities, such as anti-fungi, -bacteria, and -parasites [30-32]. Besides, PCL is a synthetic aliphatic polyester with hydrophobic properties. It is slowly degraded under physiological conditions [14, 15]. Therefore, the preparation of Chi-PCL NFs leads to amphiphilic behavior in the final sample [33].

The results of this study were comparable with other studies. For example, by mixing different Chi 1% and PCL 10% ratios, NFs with a range of 320 – 730 nm were reported [21]. In another study, by mixing Chi and PCL with a concentration of 1 and 8%, respectively, NFs with a diameter of 100-200 nm were prepared [34]. Besides, the water contact angle with the surface of PCL-Chi NFs (6:20) was reported as 120º [35]. In another study, the contact angle of PCL-Chi-gelatin NFs was 128º [36]. The mentioned contact angle values are comparable with the result of this study; a slight difference is related to the ratio of PCL and Chi or using another extra polymer.

ATR-FTIR spectra of each polymer and the Chi-PCL NFs are depicted in Figure 3. In Chi spectra, a strong band in the region 3352–3290 cm^{-1} is belonged to N-H and O-H stretching. The absorption at around 2867 cm^{-1} is related to C-H stretching; this band is characteristic of polysaccharides. N-acetyl groups' residual appeared at 1644 cm^{-1} (C=O stretching of amide) and 1316 cm^{-1} (C-N stretching of amide). An absorption band at 1588 cm^{-1} was related to the N-H bending of the primary amine. The absorption bands at 1374cm^{-1} are associated with the CH_3 bending vibration of Chi.

For the characterization of PCL powder, some band is standard. A broad and strong band in the region 3303cm^{-1} is related to OH. The absorption bands at 2942 and 2865 cm^{-1} are attributed to the PCL hydrocarbon's C-H stretching vibration. An absorption band at about 1710

cm^{-1} is related to carbonyl groups' stretching vibration (C=O stretching of ester). Furthermore, the characteristic absorption band in 1237 cm^{-1} belongs to the stretching vibration of (C–O).

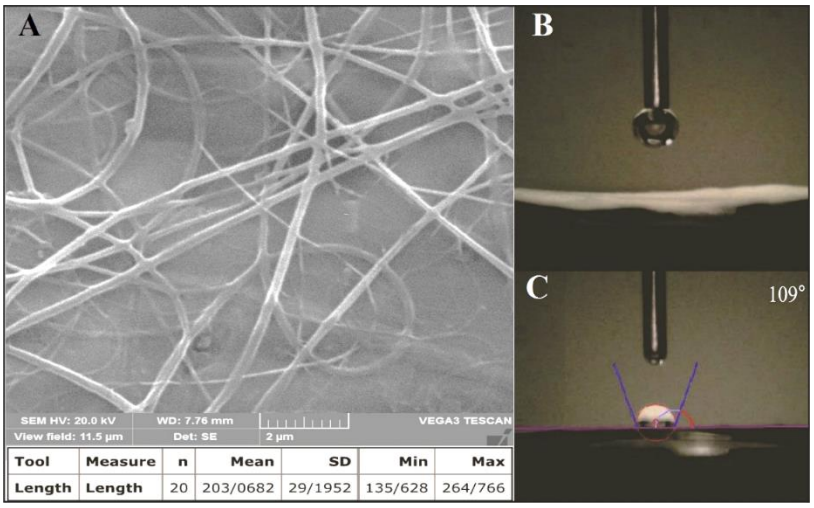

Figure 2. SEM image of prepared Chi-PCL NFs (A), Water droplet during injection (B) and after 5 s (C) for measurement of hydrophilicity of the NFs surface.

ATR-FTIR *spectroscopy of Chi-PCL NFs has* confirmed the presence of both PCL and *Chi in achieved NFs.* The broad peak at 3434 cm^{-1} belongs to the O-H and N-H groups of Chi. The specific peak at 2943 cm^{-1} showed that the C-H stretching vibration of hydrocarbon in PCL and Chi. The strong peak at 1723 cm^{-1} proves the existence of (C=O) of PCL. Also, an absorption band at 1187cm^{-1} confirmed the C-O stretching of the ester functional group of PCL.

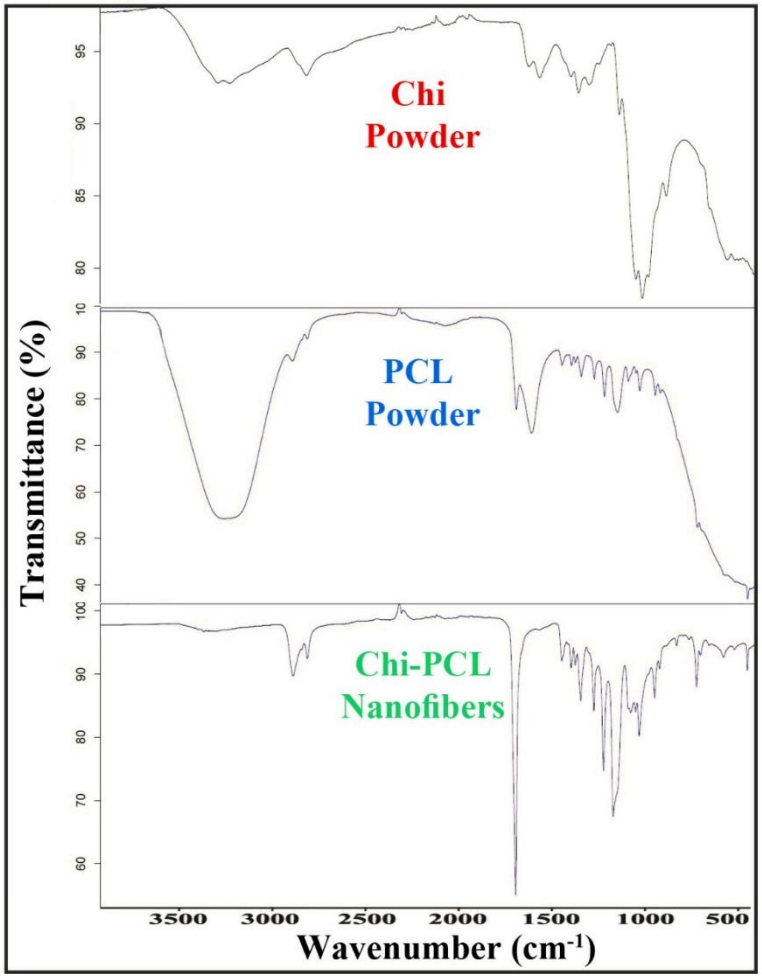

Figure 3. ATR-FTIR of Chi and PCL powders and electrospun NFs of PCL-Chi.

3.4. Prepared nanoemulsions-based nanogel and its characteristics.

Figure 4A shows that the droplet size and SPAN of the prepared emulsion were 225 ± 7 nm and 0.97 ± 0.01, respectively. Images of the nanoemulsion, nanogel, and blank gel and the effect of different shear rates on the viscosity of the nanogel are given in Figure 4B and C. A schematics of the impregnated nanogel on NFs (NFsGel and NFsGel(-oil)) is given in Figure 4D. The rheology of the nanogel follows non-Newtonian fluids; viscosity decreases by increasing the shear rate. The behavior of this nanogel is fitted with the Carreau–Yasuda model, a well-known equation for the viscosity of non-Newtonian fluids [37].

NGels have been widely used to improve the topical delivery of hydrophobic cargoes; e.g., a nanoemulsion-based nanogel of amphotericin B was prepared using carbomer 980. The percutaneous permeation flux rate (μg/cm^2/h) of nanogel (18.09±0.6) was better than nanoemulsion (15.74±0.4) and drug solution (4.59±0.01) [38, 39].

Nowadays, it is accepted that nanoemulsions with small droplet sizes have better interaction with the microorganism's outer membrane. Besides, After the disruption of walls by surfactants, the cargo's effectiveness (e.,g EO) is enhanced [40-42]. Therefore, in the current research, CSEO was first formulated into nanoemulsion. Moreover, by converting nanoemulsions to nanogels, their viscosity increases, so they could have a better accumulation on the site, which leads to better hydration of the site [43]. The nanometric EO dispersion and the better hydration of the site lead to better penetration of the EO in to the locality [44].

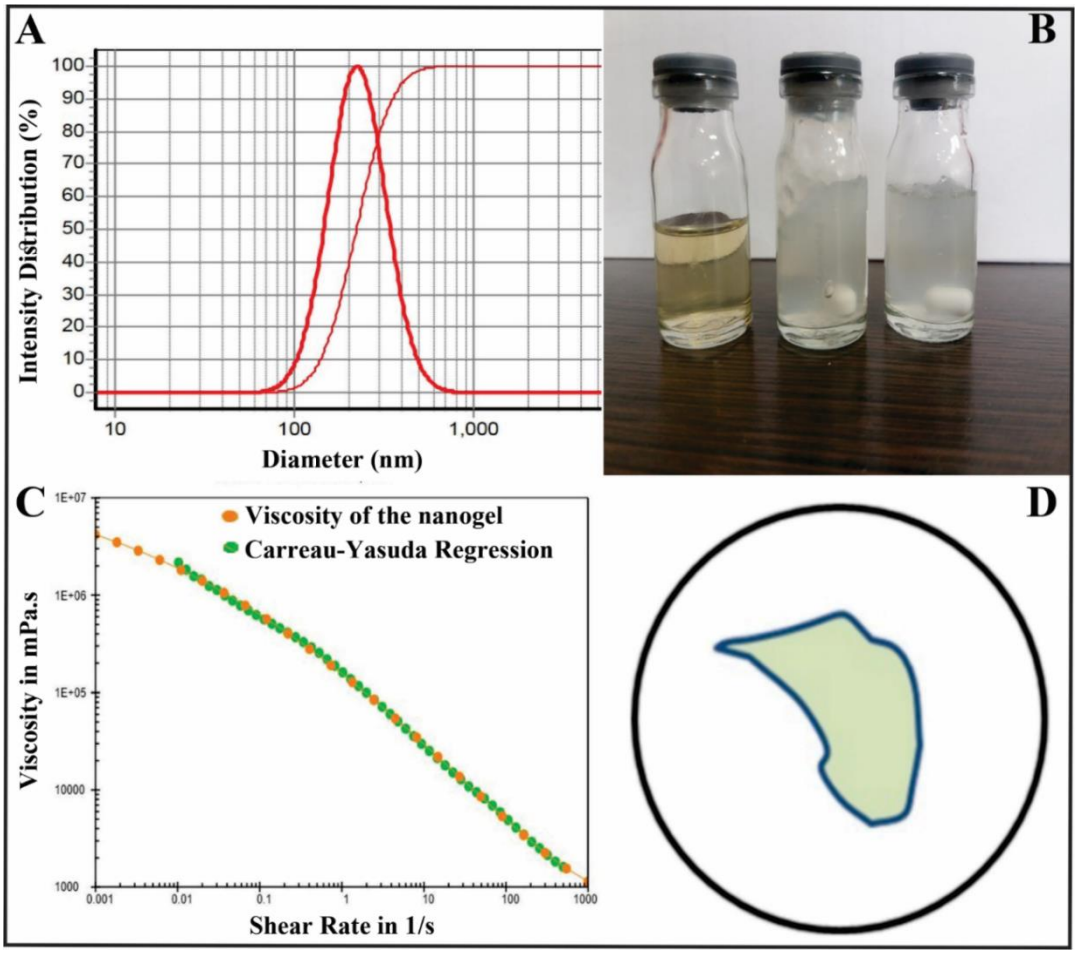

Figure 4. DLS analysis of the nanoemulsion (A), Images of the nanoemulsion, nanogel, and blank gel (B), Adaptation of viscosity of prepared nanogel to Carreau-Yasuda model (C), and schematic of impregnated nanofibers with nanogel (D).

3.5. Leishmanicidal effect of nanofiber impregnated with nanogel (NFsGel).

The leishmanicidal activities of the CSEO (80 µg/mL), NFsGel(-oil), and NFs(Gel) are demonstrated in Figure 5. By applying the NFsGel in the MTT assay, the concentration of CSEO eventually reached 80 µg/mL. Due to the leishmanicidal activity of Chi [45], NFsGel(-oil) also had a significant effect on both of *L. tropica* and *L. major* in comparison to the control group (Independent sample t-test, sig < 0.05).

Besides, the leishmanicidal effect of NFsGel was significantly better than all samples (one-way ANOVA, sig < 0.05); the viabilities of *L major* and *L. tropica* were reduced to less than 10%. Interestingly, by impregnating 11 mg of nanogel on the NFs (instead of 9.15 mg), the promastigotes' viabilities were reduced to 0%.

Some studies have been reported on using NFs or nanoemulsions as anti-leishmania agents. However, no report was found on investigating the leishmanicidal effect of impregnated NFs with nanogel. From the literature, chitosan nanofilm's therapeutic effect on cutaneous leishmaniasis has been approved in the Balb/c model [46]. In another study, by formulating of EOs of *Lavandula angustifolia* (IC_{50} 0.11 µL/mL) and *Rosmarinus officinalis* (IC_{50} 0.26 µL/mL) into nanoemulsion, their leishmanicidal effect was significantly improved against *L. major* (IC_{50}=0.08 µL/mL).

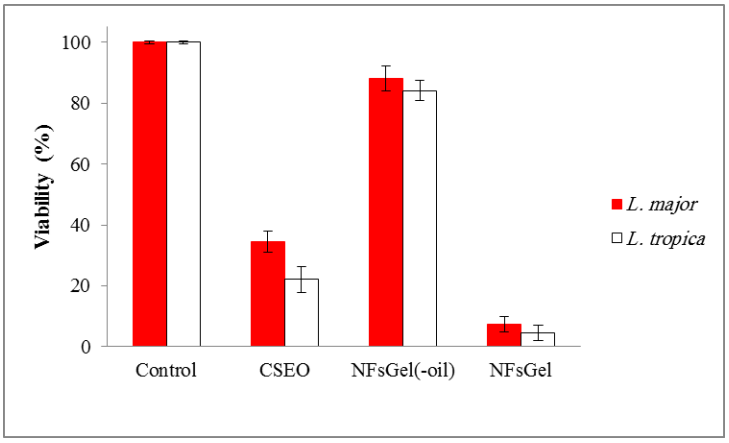

Figure 5. Leishmanicidal effect of CSEO, NFsGel(-oil), and NFsGel.

4. Conclusions

In this study, the leishmanicidal activity of CSEO was investigated. Its potency, stability, and easy usage in a topical manner were improved by preparing the nanoemulsion-based nanogel. Besides, by impregnating CSEO nanogel on the surface of Chi-PCL NFs (NFsGel), its efficiency was improved. The leishmanicidal effect of NFsGel was significantly better than the CSEO; it was reduced the viabilities of *L. major* and *L. tropica* to 0%. NFs with nanometric meshes could also prevent the entry of environmental pathogens into the lesion and secondary infection. In this system, the amount of impregnated nanogel on the NFs mat could easily be adjusted as needed. Easy packaging and storage are other benefits of this system. The prepared prototype could be used as an excellent substance for *in-vivo* studies.

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Conflicts of Interest

The authors declare no conflict of interest.

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