The wound-healing effect of *turbinaria triquetra* extract and polysaccharide contents alone or loaded on chitosan nanoparticles in human fibroblast cells: in-vitro study

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Background/aim
Seaweeds contain biologically active molecules that are important in the pharmaceutical industry. The present study aimed to evaluate the wound-healing effect of seaweed *Turbinaria triquetra* extract and polysaccharides (PE) content alone or loaded on chitosan nanoparticles (CSNPs) in human fibroblast cells [human skin fibroblast (HSF)].

Materials and methods
T. triquetra were collected from Hurghada, Red Sea, Egypt. The preparation of ethyl acetate extract (EAE) and PE was carried out. Chitosan nanoformulation was prepared using nanoparticle techniques of Dynamic Light Scattering and the Electron Microscope was used for morphology determination. The HSF cell line used was divided into a control group (untreated cell), cells treated with CSNPs, EAE, or PE alone, or loaded on EAE-CSNPs or PE-CSNPs. MTT assay was used to determine the cytotoxic effect with different concentrations of 0.1, 1, 10, and 100 μg/ml for 48 h. The healing scratched area change was measured for all groups at 0 time, after 1, 2, and 3 days with IC50 of all treatments and was confirmed by Confocal Laser Scanning Microscope.

Results
All nanoparticles revealed spherical shape and the average particle sizes, zeta potential, and polydispersity index between all groups showed significant changes ($P<0.05$); however, EAE-CSNPs was the best. The viability of the HSF cell line was assessed in treated groups and the high proliferation rate was in EAE-CSNPs and PE-CSNPs at a dose of 0.1 or 100 μg/ml. In addition, the carrier group exhibited no cytotoxic effect of *T. triquetra* extracts. The wound-healing assay revealed that the PE-CSNPs group has a strong topical healing effect on the third day of treatment. In parallel, the result examined by the Confocal Laser Scanning Microscope showed the highest proliferation in HSF cells in both EAE-CSNPs and PE-CSNPs-treated cells, while PE-CSNPs was the best and appeared with a low number of dead cells.

Conclusion
This study concluded that *T. triquetra* extract and PEs content alone or loaded on CSNPs showed an active wound-healing effect in human fibroblast cell, in addition cell treated with PEs extract of *T. triquetra* and loaded on CSNPs have a strong topical healing effect and might be a potential therapeutic agent for skin wound-healing.

Keywords:
chitosan nanoparticles, confocal, macroalgae, *Turbinaria triquetra*, wound-healing

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Introduction

Skin is the first line of protection against infections. That is why it is so important to heal wounds on the skin [1]. Healing a skin wound is a complex and dynamic process with three overlapping phases: inflammation, proliferation, and maturation, which are commonly referred to as remodeling [2]. The proliferation of granulation tissue occurs through angiogenesis and the immigration of keratinocytes and fibroblasts to the wound site. The maturation phase involves the restoration of the skin barrier and the repair of the granulation tissue within the scar, along with vessel regression [3]. This newly formed tissue matures over time with greater tensile strength [4]. Although collagen production gradually decreases after 3 weeks, cross-linking and reorganization of collagen occur in the last phase of healing [5].

Several factors affect one or more phases of the wound-healing process, and the steps of each phase must be performed accurately and regularly. Interruptions or prolongations of the process can lead to the development of chronic wounds or delayed wound-healing. Most chronic ulcers are associated with ischemia, diabetes, and hypertension [6]. Nowadays, to improve the quality and speed of the healing process, synthetic drugs such as nitrofurazone, gentamicin, mupirocin, and others are used, but these drugs have side effects. Therefore, in recent years, natural medicines have become increasingly used [7].

There are many medicinal plants with a long history of medicinal effects on various diseases. Macroalgae or seaweeds are one of the most important marine organisms. Natural products from algae have been widely explored for a long time, for human use as food and as medical treatments [8]. Several investigations explored the potential advantages of natural products on wound-healing, due to their antioxidant, antimicrobial, and anti-inflammatory qualities [8–10]. Many chemicals and products from algae have considerable economic importance and are broadly used as they are a good source of minerals, antioxidants, vitamins, pigments, steroids, lectins, halogenated compounds, carotenoids, dietary fiber, polyphenols, enzymes, polysaccharides (PE), proteins, polyunsaturated fatty acids and other lipids [11]. These compounds demonstrate antimicrobial, antioxidant, anticancer, antihelminthic, anti-inflammatory, antiaging, and cancer-cytotoxic agents [12]. For this reason, marine algae are a promising potential source of highly bioactive secondary metabolites that could serve as an effective starting point in the development of many industries of diverse branches, such as food, fuel, plastics, cosmetics, and pharmaceuticals, which are highly beneficial [13].

Nanotechnology is one of the promising scientific directions of the 21st century. It is intended to manipulate the matter at the nanometer scale, which is about 1–100 nm, to different technical and biomedical applications. However, the term nanoscience began with an interest speech entitled "There's Plenty of Room at the Bottom" on December 29, 1959, by physicist Richard Feynman during the American Society meeting at the California Institute of Technology. The term "nanotechnology" was first introduced by Professor Norio Taniguchi in 1974 to describe the ability to develop novel features via controlling the arrangement of atoms and molecules at the nanoscale [14]. Drug carriers based on nanotechnology are more advantageous for health than conventional formulations. Nanotechnology is used to create therapeutic agents that are then placed in biocompatible nanocarriers such as carbon nanotubes, micellar systems, liposomes, dendrimers, nanoparticles (NPs), and nanocapsules [15]. In order to achieve improved specificity, drug targeting, and delivery efficiency, as well as to achieve a maximal therapeutic effect with a minimum of side effects, nanocarriers (typically ranging from 1 to 1000 nm and suitable for the delivery of drugs, hormones, genes, nucleic acids, or imaging agents) have been developed [16]. Chitosan nanoparticles (CSNPs) have been used in the field of drug delivery, tissue engineering, and other biomedical applications [13].

On the other hand, *Turbinaria triquetra* (brown algae) is a marine macroalgae or seaweed that is recognized to have health impacts and found in abundance in the Red Sea [12]. The current study aimed to investigate the wound-healing capacity of brown macroalgae *T. triquetra* extract and PEs content alone or loaded with CSNP on human fibroblast cells [human skin fibroblast (HSF)].

Materials and methods

Chemicals

Analytical grade (methanol, ethanol), chloroform, n-butanol, ethyl acetate, 2,2-Diphenyl-1-picrylhydrazyl, ascorbic acid, gallic acid (Sigma-Aldrich Germany), standard Folin–Ciocalteu's phenol reagent (SD Fine-Chem Limited, Mumbai, India), Tris (hydroxyl methyl) amino methane hydrochloride (Merck Ltd., Cairo, Egypt), and sodium carbonate. Chitosan (CS) of Mw 190–310 kDa and a deacetylation degree of 75–85% was obtained from Golden-Shell Biochemical Co. Ltd (Hangzhou, China). Sodium tripolyphosphate (STPP) was purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Glacial acetic acid was commercially available and used as received. Distilled water was used in the experiment.

Dulbecco's modified Eagle's medium (#AL219A, Himedia India), fetal bovine serum (#RM10432, Himedia India), Delbucco's phosphate buffered saline (#TL1006, Himedia India), mouse anti-human collagen IFluorescein isothiocyanate antibody (#FCMAB412F, Merck Ltd., Cairo, Egypt), Cipladine (Cipla Ltd), hEGF (#11376454001, Roche Basel, Switzerland) MTT Reagent (# 4060 Himedia India), DMSO (#PHR1309, Sigma Germany), Fluorescent Activated Cell Sorter (FACS) Calibur (BD Biosciences, India), Microplate reader (#EC800, Biotek, India).

Brown alga collection and identification

T. triquetra (J. Agardh) was collected in autumn (2017) from Hurghada, Red Sea, Egypt. Based on the catalog of Braun and Guiry [17] and Guiry and Guiry [18], Prof. Dr Nihal Galal El-Din Shams El-Din, Professor in Hydrobiology Laboratory, National Institute of Oceanography and Fisheries (NIOF), Cairo, Egypt identified the samples. *T. triquetra,* Class Phaeophyceae, Order Fucales, Family Sargassaceae. A voucher specimen (M.AL.1) was deposited in the herbarium of the National Research Centre, Cairo, Egypt (CAIRC) by Prof. Dr Mona M. Marzouk. Fresh samples were thoroughly washed with seawater and epiphytes were removed. Samples were air dried at room temperature in shade, then milled to a fine powder for further analysis.

T. triquetra extraction

Preparation of crude extract and fractionation

The preparation of crude extract and fractionation of *T. triquetra* was done from the powder sample, which was extracted by the sonication method and fractionated by partitioning as previously mentioned [19]. The ethyl acetate fraction was collected and evaporated till dry, yielding a sticky reddish brown fraction [ethyl acetate extract (EAE), 3.86 g].

Polysaccharide extraction

The remaining algal residue was dried at room temperature and subjected to PE extraction, according to Mettwally *et al.* [20]. The PE was extracted using the hot water technique and precipitated using cold ethanol and refined by deproteinization yielding 11.36 g.

Chitosan nanoparticles synthesis

CSNPs and CS loaded with PS EAE loaded on CSNPs, semipurified PEs loaded on CSNPs were prepared as previously mentioned by Abdel-Aal *et al.* [21]. The starting CS solution was decreased to 0.1% (w/v). The prepared nanoparticles were characterized using techniques such as Dynamic Light Scattering to determine the size and size distribution. The morphology of the nanoparticles was observed using Transmission Electron Microscopy.

Experimental design

The HSF cell line used was divided as follows:

(1) Control group: untreated cells.
(2) CSNPs group: carrier cell with CSNPs.
(3) EAE group: cells treated with EAE of *T. triquetra*.
(4) PE group: cell treated with semipurified PEs extract of *T. triquetra*.
(5) EAE-CSNPs group: cells treated with EAE of *T. triquetra* and loaded on CSNPs.
(6) PE-CSNPs group: cells treated with semipurified PEs extract of *T. triquetra* and loaded on CSNPs.

MTT assay was used to determine the cytotoxic effect with different concentrations of 0.1, 1, 10, and 100 µg/ml for 48 h. The healing scratched area change was measured for all groups at 0 time, after 1, 2, and 3 days with IC50 of all treatments and was confirmed by Confocal Laser Scanning Microscope (CLSM).

Methods

Cell line, cell culture propagation, and maintenance

HSF cell line was purchased from NAWAH Scientific Research Centre. The following operations were all carried out in a sterile setting while employing a Class II A2 Laminar Flow Biosafety Cabinet (Manufactured by Labconco). Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum (Gibco, USA), 100 µg/ml penicillin, and 100 µg/ml streptomycin sulphate (Lonza, Belgium) was used to maintain cells at 37°C in a humid incubator with 5% CO_2. The cells were subcultured using a trypsin-EDTA solution (0.025% trypsin and 0.02% EDTA) once they had achieved 70–90% confluence (Lonza). Cells were taken out at the logarithmic stage of growth for each experiment. The tested substances were dissolved in distilled water and kept at −20°C for storage. Before each experiment, stock solutions were diluted with culture medium for the final dosage [9].

Evaluation of cytotoxicity by MTT assay

According to Mosmann [22], the MTT test was used to measure the dye cytotoxicity against HSF cells. In order to generate a semiconfluent layer, cells were seeded in a 96-well microplate at a density of 1×10^4 cells/well in 100 µl of complete growth media. They were then incubated at 37°C and 5% CO_2 overnight. For 48 h, cells were exposed to the medicines at successive dilutions with final concentrations of (100, 10, 1, 0.1 µg/ml). Complete growth media was added in place of the tested medications for the cells that were not treated (the negative control). Following the incubation period, the medium was removed, the cells were washed with PBS, and 100 µl of MTT (0.5 mg/ml) was added to each well. The wells' serum-containing media were still present when this was done. In order to dissolve the generated formazan crystals, 100 µl of DMSO was added to each well before being well mixed. Using a microplate reader, at 492 nm and a reference wavelength of 630 nm, the

optical density (OD) of each well was measured (Model 4300; Chromate Instrument, Awareness technology Inc., Palm City, Florida, USA). OD test/OD control/100 was used to calculate the percentage of cell viability. Using the Sigma Plot software, version 11, a four-parameter logistic curve (log concentration vs. %cell growth as compared to control cells) was used to calculate the IC50 value of the tested substances (concentration of sample causing a 50% loss of cell proliferation of the vehicle control). %Viability=absorbance of drug/absorbance of control×100.

Wound-healing assay

To study cell migration and wound-healing, the "in-vitro scratch assay" method was performed. HSF cells were seeded in a 6-well plate with 4×10^5 cells per well and incubated for 24 h. After this time, all medium was withdrawn and all cells were allowed to starve in culture medium containing 1% fetal bovine serum and 1% PS for 24 h. Then, the scratch was made in each well with a yellow tip and photographed as time 0 with an inverted microscope with ×10 magnification. All medium was withdrawn and replaced with treatments with a concentration of IC50 (186, 208.17, 203.14, 287.92, and 129.58 for EAE, PE, EAE-CSNPs, PE-CSNPs, and carrier, respectively). Fresh medium was served as a control. Plates were further incubated and images were recaptured at the same locations after 24, 48, and 72 h. The percentage increase in wound closure compared to the value obtained before treatment was measured and reported as wound-healing [23].

Confocal Laser Scanning Microscopy analysis of live/dead

CLSM (DMI 8 Leica, Germany) was used to study the proliferation effect of treatments on HSF at concentrations of IC50 (186, 208.17, 203.14, 287.92, 129.58 for EAE, PE, EAE-CSNPs, PE-CSNPs, and carrier, respectively), for discrimination of live from dead cells on the basis of membrane integrity. Acridine orange fluoresces green (AO) (Sigma-Aldrich, USA) was used to stain live cells, while propidium iodide fluoresces red (PI) (Sigma-Aldrich) was used to stain dead cells. In brief, HSF cells were seeded in 3.5 petri dishes with 4×10^5 cells each and incubated for 24 h. After this time, all medium was withdrawn and replaced with treatments with concentrations (IC50) and cells were incubated for 48 h and then 200 µl of AO/PI (1 mg/ml) were added and left for 15 min, then the samples have been examined immediately under confocal microscopy using the excitation laser lines at 488 and 552 nm [24].

Statistical analysis

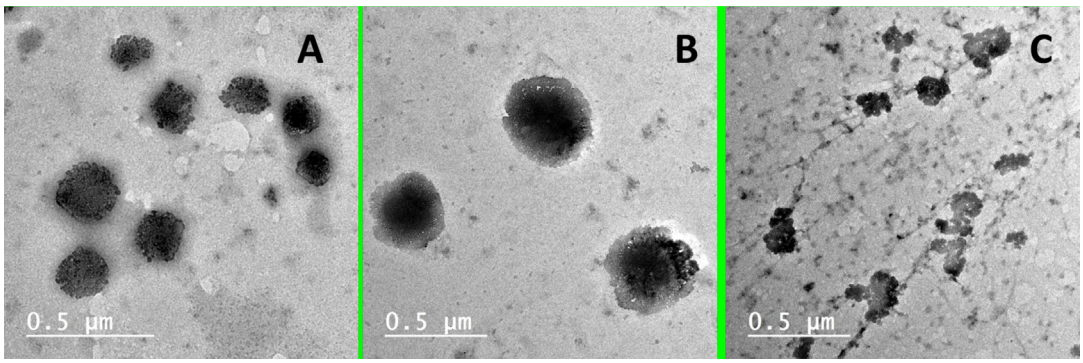
IBM SPSS statistics (Statistical Package for Social Sciences) software, version 22.0, IBM Corp., Chicago, Illinois, USA, 2013, was used. Descriptive statistics were done as mean±SE for quantitative parametric data. Differences between groups were evaluated using one-way analysis of variance (ANOVA) with post-hoc Tukey honest significant difference. Differences were regarded as statistically significant at P value less than 0.05.

Results

Microstructure characterization of the formed nanoparticles

The data illustrated in Fig. 1 showing Transmission Electron Microscopy of CSNPs (A), PS-CSNPs (B), and EAE-CSNPs (C). All nanoparticles revealed spherical shape, and the average particle sizes of the fabricated nanoparticles were 291.6±51.3 nm for CSNPs and 360.5±33.7 nm for PS-CSNPs and 143.8±65 mm for EAE-CSNPs based on Dynamic Light Scattering measurements, as shown in Table 1. Moreover, the statistical analysis showed

Figure 1



TEM micrographs of (a) CSNPs, (b) PS-CSNPs, and (c) EAE-CSNPs. CSNPs, chitosan nanoparticles; EAE, ethyl acetate extract; PS, polysaccharides; TEM, Transmission Electron Microscopy.

Table 1 Average particle size, zeta potential, and polydispersity index of CSNPs, PS-CSNPs, and EAE-CSNPs based on DLS measurements

Nanoparticles samples	Average particle size (nm)	Zeta potential (mV)	PDI
CSNPs	291.6±51.3[a]	18.1±2.3[a]	0.45[a]
PS-CSNPs	360.5±33.7[b]	26.4±4.9[b]	0.29[b]
EAE-CSNPs	143.8±65[c]	10.4±1.8[c]	0.51[a]

CSNPs, chitosan nanoparticles; EAE-CSNPs, ethyl acetate extract loaded on chitosan nanoparticles; PDI, polydispersity index; PS-CSNPs, semipurified polysaccharides loaded on chitosan nanoparticles; All data are presented with different letter (a, b, c) within the same column are significant using analysis of variance test.

significant changes ($P<0.05$) in average particle size, zeta potential, and polydispersity index between all groups and the EAE-CSNPs group was the best.

Cytotoxicity (MTT)

The viability of the HSF cell line was assessed in all treated groups with different concentrations of 0.1, 1, 10, and 100 µg/ml of administration. The high proliferation rate was shown in EAE-CSNPs and PE-CSNPs at a dose of 0.1 or 100 µg/ml, as shown in Fig. 2. The EAE, PE, EAE-CSNPs, and PE-CSNPs exhibited a viability of 98.78, 107.73, 134.02, and 142.1%, respectively, at a dose of 100 µg/ml, indicating its safety on HSF cells, especially PE-CSNPs with 142.13%, and while at 0.1 showed more 72.07, 84.17, 90.42, and 95.64%, respectively, indicating the high proliferation rate, especially in EAE-CSNPs and PE-CSNPs.

Wound-healing

The results of the scratch wound-healing assay in carrier, EAE, PE, EAE-CSNPs, and PE-CSNPs groups of HSF cells indicated that PE-CSNPs have a strong topical healing effect after 3 days of treatments, as illustrated in Fig. 3. using an inverted microscope. The findings showed that PE-CSNPs increased fibroblast migration in mechanically produced wounds at a concentration of IC50. The wound-healing image showing closure of the wound was observed faster and almost completed more than other groups, as shown in Fig. 4.

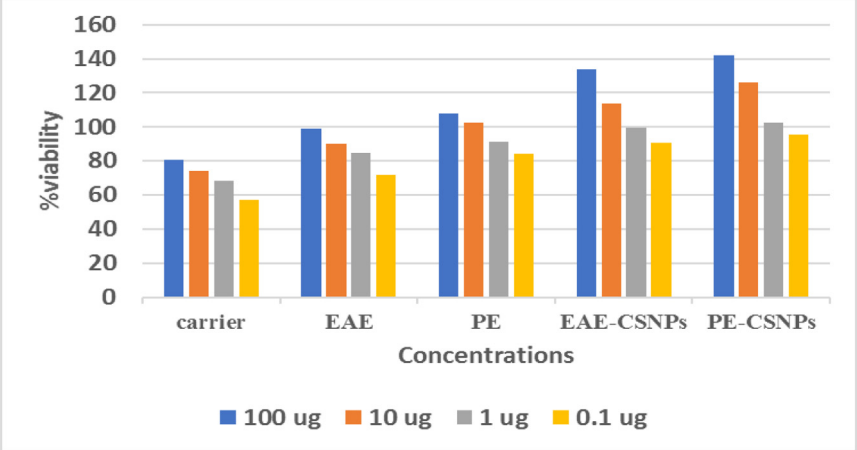
Confocal Laser Scanning Microscope

Figure 5 represents the distribution of dead/viable cells in the tested concentrations of IC50 dose, where the viability of the cells appear in green fluorescence for live HSF cells and red fluorescence appeared as a result of dead abundance, while yellowish orange fluorescence due to the cross over between the green (live cells) and red (dead cells) with the same extent. However, the result of CLSM imaging of HSF exhibited that the laser disintegrates the HSF cells in EAE-CSNPs and PE-CSNPs groups and showed the highest proliferation, and the PE-CSNPs group was the best when compared with the control group.

Discussion

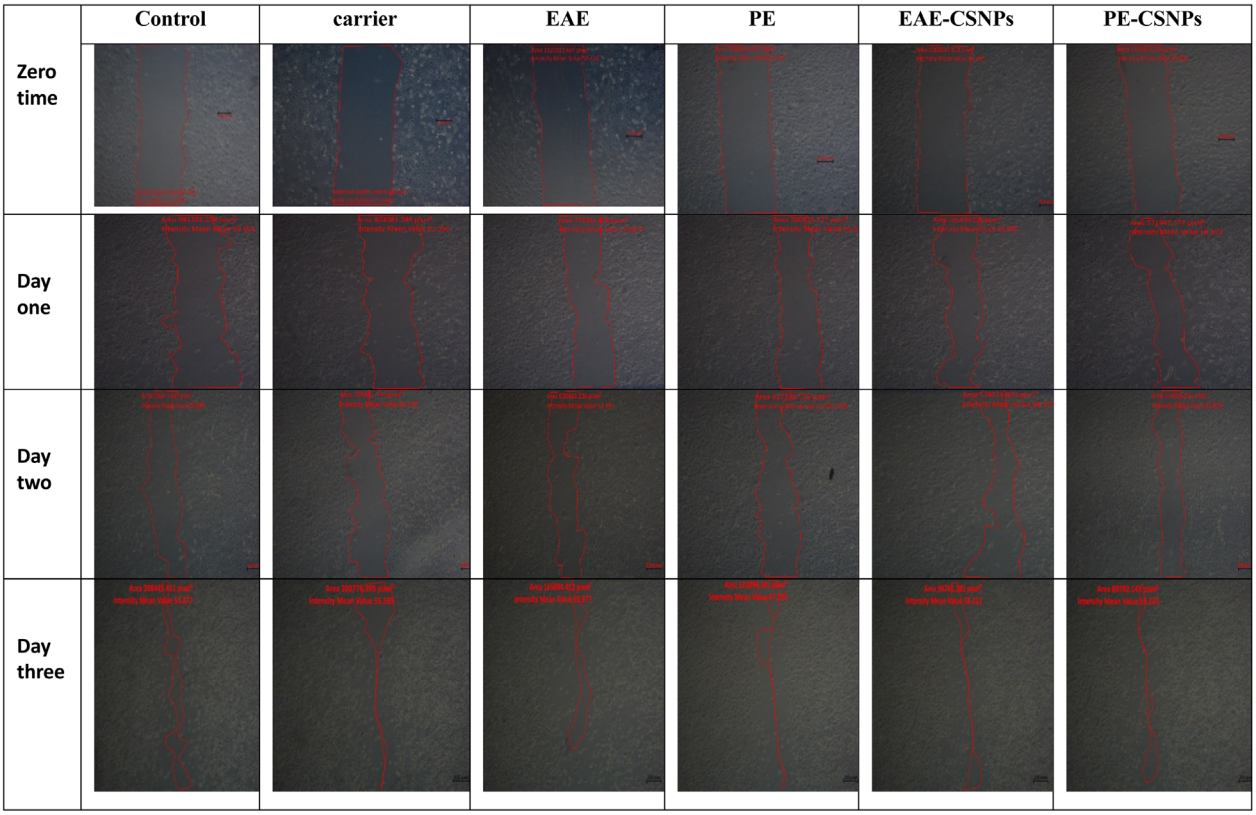
Macroalgae has become a priority selection for wound care and treatment due to its advancements in promoting healing wounds [11]. With the rapid development of modern technology, more and more new potential biomaterials have been created and have been intensely exploited for wound-healing applications [2]. On the other hand, the promising CSNPs were achieved to improve specificity, drug targeting, and delivery efficiency; moreover the maximal therapeutic effect with a minimum of side effects [14,15]. However, the current study is aiming to evaluate the effect of *T. triquetra* (brown algae) extract and PEs content alone or loaded on CSNPs on the HSF cell line.

Figure 2



The cell viability percentage of HSF cell line after all treatments with different concentrations. HSF, human skin fibroblast.

Figure 3

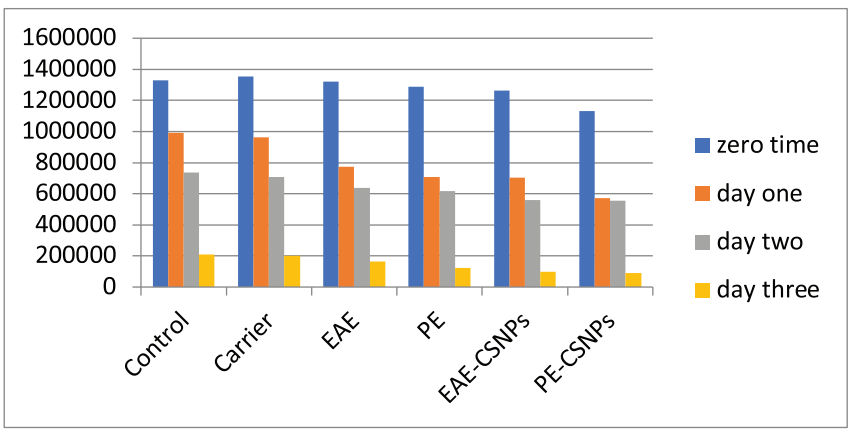


Photographs of wound-healing of control HSF cell and all treated groups with IC50 dose at 0, 1, 2, and 3 days, using inverted microscope (×10 magnifications). HSF, human skin fibroblast.

The HSF cell line used in this study was divided into a control group (untreated cell), cells treated with CSNPs, EAE of *T. triquetra*, PEs content, alone or loaded on EAE-CSNPs and PE-CSNPs. MTT assay was used to determine the cytotoxic effect with different concentrations of 0.1, 1, 10, and 100 µg/ml for 48 h. The healing scratched area change was measured for all groups at 0 time, after 1, 2, and 3 days with IC50 of all treatments and was confirmed by CLSM.

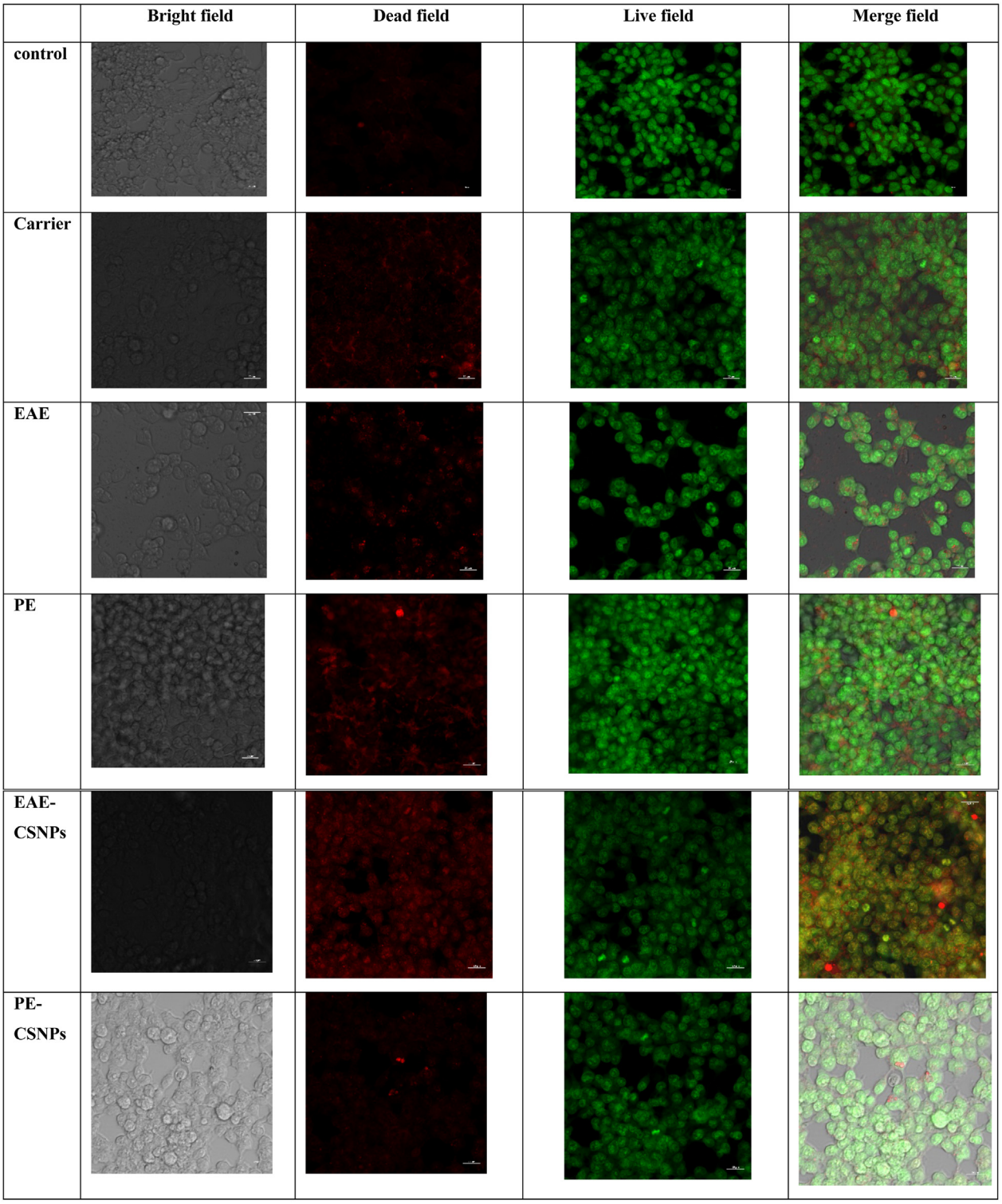
The present study shows that all nanoparticles of CSNPs, PS-CSNPs, and EAE-CSNPs revealed spherical shape, and the average particle sizes of the fabricated nanoparticles were 291.6±51.3 nm for CSNPs and 360.5±33.7 nm for PS-CSNPs and

Figure 4



The wound-healing scratched area of control HSF cell and all treated groups with IC50 dose at 0, 1, 2, and 3 days. HSF, human skin fibroblast.

Figure 5



Confocal laser scanning microscopic photographs for viable/dead HSF cell line stained with acridine orange (green fluorescence) and propidium iodide (red fluorescence) using IC50 dose (magnification, ×40). HSF, human skin fibroblast.

143.8±65 mm for EAE-CSNPs. Moreover, the statistical analysis showed significant changes in average particle size, zeta potential, and polydispersity index between all groups. Previously it was found that the biocompatibility of CSNPs makes them an attractive option for developing advanced therapeutic systems [13], however the formation of CS-tripolyphosphate (TPP) nanoparticles is based on the ionic gelation method, where chitosan, a natural PE derived from chitin, is cross-linked with TPP which is negatively charged and interacts with the chitosan through electrostatic interactions, leading to

the formation of nanoparticles. The size of the nanoparticles can range from 50 nm to several hundred nanometers, depending on the concentration of chitosan and TPP, as well as other factors like pH and ionic strength. These nanoparticles are typically spherical and can be designed to have a narrow size distribution [14,15].

The viability of the HSF cell line was assessed to identify the treatments potential cytotoxicity, which could provide important information about the safety of these active chemicals when applied topically [10]. In this study, no cytotoxic effect of *T. triquetra* extracts and chitosan nanoformulations on HSF cell line were observed., The high proliferation rate was observed in HSF cell treated with EAE or with PEs content that are loaded on EAE-CSNPs and PE-CSNPs, respectively at a dose of 0.1 or 100 µg/ml and the effect of PE-CSNPs was the best, in addition the carrier group exhibited no cytotoxic effect. Additionally, the high efficient of PE-CSNPs on the scratch wound-healing of HSF cells was ascribed to the high antioxidant activity of this new nanoformulation of the algae, which increases the inhibition effect of wound infection and improves the fast healing of wound scratch [21].

The current results of the scratch wound-healing assay showed that HSF cells treated with PEs extract of *T. triquetra* that were loaded on CSNPs, have a strong topical healing effect on the third day of treatment. In parallel with the result examined by the CLSM, which exhibited a remarkable laser disintegration and showed the highest proliferation in HSF cells in both EAE-CSNPs and PE-CSNPs-treated cell, while PE-CSNPs was the best and appeared with a low number of dead cells. The present results agreed with Deyab *et al.* [25], who reported the strong relation between the increasing of brown algae consumption and human diseases prevention could be explained by their content of antioxidants which include phenolics, alkaloids, flavonoids, coumarins, steroids, and PEs such as alginic acid, fucoidan, laminarin, and mannitol. Also the same results were found in other species of brown algae, where Algal sulfated PEs extracted from *Sargassum fusiforme*, *Graciaria lemaneiformis*, and *Laminaria japonica*, respectively, were used to prepare hydrogels together with chitosan and polyvinyl alcohol. The three hydrogels significantly increased the wound-healing effects compared with those of the conventional hydrogels [26].

Conclusion

The present study concluded that *T. triquetra* extract and PEs content alone or loaded on CSNPs showed an active wound-healing effect on human fibroblast cell, which was the first research in addition cell treated with PEs extract of *T. triquetra* and loaded on CSNPs have a strong topical healing effect and might be a potential therapeutic agent for skin wound-healing. Further studies are highly warranted to identify active molecules in the extracts. Moreover, in-vivo studies would further verify wound-healing therapeutic applications.

Author contributions: all authors have substantially contributed to the conception and design of the study. The drafting of the article or revising it critically for important intellectual content, and giving final approval of the article for submission.

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Conflicts of interest

There are no conflicts of interest.

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