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Research article

In Vitro Evaluation of the Wound Healing Properties and Safety Assessment of Fucoidan Extracted from *Sargassum angustifolium*

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Abstract

Keywords

Sargassum angustifolium;

adipose derived mesenchymal stem cells;

partially purified extract;

fucoidan;

wound healing

Seaweeds are rich in fucoidan, which has various biologic effects. So far, a lot of research has been conducted on the biological effects of fucoidan extracted from various species of seaweed. However, the effects of partially purified extract of *Sargassum angustifolium* (PPE-SA) on wound healing (cell migration) of adipose-derived mesenchymal stem cells (ADMSCs) have not yet been studied. In this experimental study, crude fucoidan was extracted from *S. angustifolium* using an advanced method. After removing lipids, pigments and low molecular weight compounds with ethanol and removal of alginate with $CaCl_2$, polysaccharides in the remaining material were extracted with hot water (60ºC). The polysaccharides of the resulting extract were precipitated with ethanol. Then, the wound healing and safety of PPE-SA on ADMSCs were investigated by MTT and the scratch assay, respectively. The MTT assay showed that PPE-SA did not only have a negative effect on the growth of mesenchymal cells but at some concentrations improved their growth by up to 1.5 times. The PPE-SA also increased ADMSCs migration by 76% and 142% after 48 h and 72 h incubation, which showed the superiority of this seaweeds extract over many other reported species and genera. The results of this study showed that an extract of this seaweed obtained by this method has the potential to promote the growth of mesenchymal stem cells and may have a high potential for use in tissue engineering applications.

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1. Introduction

Wound healing is a complex process. It involves cellular, molecular, and physiological interactions that ultimately lead to the regeneration and replacement of damaged tissue. Inflammation and blood clot formation, which occur immediately after injury, are the first steps in the wound healing process. After four days of injury, the inflammatory response is accompanied by the proliferation and migration of dermal and epidermal cells. At this stage, a cellular matrix is created to fill the wound gap and rebuild the skin barrier. Finally, the wound healing process is completed with the maturation and remodeling of the damaged tissue. From ancient times, plants have been used as medicines to accelerate the healing process. They have been prescribed for different types of injuries including cuts and burns.

Algae extract is rich in various substances, and one of them with the capability to enhance the interior processes of stem cell migration is fucoidan. It is a plentiful and cost-effective marine polysaccharide that possesses a wide range of biological properties, including anti-clotting, anti-thrombotic, anti-viral, and anti-inflammatory properties [1]. Fucoidan contains high percentages of L-fucose and sulfate ester groups, which exists in the amorphous section of the brown seaweed cell walls, and also in some marine invertebrates such as sea urchins and sea cucumbers. Some studies have shown that low molecular weight fucoidan has a healing effect on skin wounds [2]. Up to now, numerous studies have been conducted on fucoidan extraction from different sources [3], and it has been characterized as having anticancer activity [4], anti-virus activity, cell mobilization potential [5-8] and immunostimulatory activity [9]. However, there has been no report on the effects of partially purified extract (water/ethanol extract) of *S. angustifolium* (PPE-SA) on tissue regeneration and wound healing. Therefore, the main aim of our experiment was to reveal the biological effect of water/ethanol extracts of *Sargassum*. Premarathna *et al.* [10] studied the effects of aqueous extracts of *Sargassum* on wound healing [10], but in our experiment, we used water/ethanol extracts to find out whether there was a significant difference between these two methods of extractions in terms of their biological effects. The results showed that there was a great difference between the biological effect of these two types of extracts. In the present experiment, the water/ethanol extract at a concentration of 0.20 µg/µL (200 µg/mL) had the same effect as 7.79 µg/µL of the aqueous extract reported by Premarathna *et al.* [10].

2. Materials and Methods

2.1 Chemicals

Sargassum angustifolium was purchased from the Algae Bio Resource Development Company, Shiraz, Iran (voucher number: abdfm11114). ADMSCs were purchased from the Stem Cell Technology Research Center. MTT and Standard L-fucose (f 2252) were purchased from Sigma-Aldrich. All chemicals used were of analytical grade and used as received.

2.2 Isolation of partially purified extract (crude polysaccharide) *from S. angustifolium* (PPE-SA)

The extraction of the crude polysaccharide (crude fucoidan) (PPE-SA) was performed according to the method of Borazjani *et al.* [3]. In this method, after removing lipids, pigments and low molecular weight compounds with ethanol and removal of alginate by $CaCl_2$, polysaccharides in the resulting algae material were extracted with hot water (60ºC). Then, the polysaccharides in the resulting extract were precipitated with ethanol [3]. Accordingly, the following steps were followed: brown

seaweed was milled for 75 min at 4000 rpm in a planetary ball mill (Germany). Fifteen grams of powder was mixed with 350 mL of 99% ethanol and stirred for 12 h at room temperature. It was then centrifuged at 2000 rpm for 10 min. The precipitate was kept at room temperature to remove its ethanol. Subsequently, 0.5 g of the resulting powder was mixed with 100 mL of distilled water and stirred for 1 h at 65°C, then centrifuged for 14 min at 11000 rpm. $CaCl_2$ (0.5 g) was added to the solution. The supernatant was collected and kept for 12 h at 4°C. After that, the solution was centrifuged at 11000 rpm for 14 min. The supernatant was mixed with 99% ethanol to reach 30% ethanol concentration. It was incubated at 4°C for 4 h. After centrifugation for 14 min at 11000, the supernatant was brought to a 70% ethanol concentration and incubated for 4 h at 12°C. Then it was centrifuged at 10000 rpm for 10 min. The resulting sediment was powdered in a freeze-dryer [11].

2.3 Extract characterization

2.3.1 SEM image

The surface morphology of PPE-SA was analyzed by scanning electron microscopy (MIRA III, TESCAN, Czech Republic). The samples were attached to 10 mm metal mounts using double-sided adhesive tape and sputter-coated under a vacuum atmosphere with gold.

2.3.2 FTIR spectra

To measure the FTIR spectra, a Fourier transform infrared spectrometer (AVATAR, Thermo, USA) was used in the range of 4000 to 400 cm^{-1} at room temperature.

2.3.3 ICP-OES analysis

The chemical element concentration of the brown algae extract (20 mg dispersed in 20 mL distilled water) was analyzed using a Varian (Inc., Melbourne, Australia) Vista Pro (MPX) radial inductively coupled plasma atomic emission spectroscope (ICP-OES) instrument.

2.3.4 CHNS analysis

Carbon, hydrogen, nitrogen and sulfur content were evaluated with a CHNS analyzer (LECO-TruSpec Micro USA). The degree of sulphation (DS) of the PPE-SA was calculated by elemental analysis according to the following formula. For this purpose, all carbohydrate residues in the PPE-SA were considered to be hexose.

$$S\ (\%)/C\ (\%) = (32.06 \times DS)/\ (12.01 \times 6) \quad (1)$$

2.3.5 L-fucose assay

The L-fucose content of the samples was calculated by Dische and Shettles' method [12] with some modifications. Firstly, 2 mg/mL of PPE-SA samples were prepared and placed in an ice-water bath. After that, 4.5 mL of dilute sulfuric acid (6:1, H_2SO_4:H_2O) was added to each sample. The samples were placed in ice for 1 min, and then placed in a boiling water bath for 10 min. After reaching room temperature, 0.1 mL of 3% L-cysteine was added to each sample. The solutions were mixed and allowed to stand for 30 min before measuring the absorbance at both 396 nm and 427 nm. The values of (A396 nm-A427 nm) was used to exclude hexoses, as methyl pentose absorbance value were the objective. Standard L-fucose was used for constructing the standard curve via the same procedure.

2.4 Cytotoxicity assay

A cytotoxicity evaluation of PPE-SA was performed by 3-(4, 5-dimethyl thiazol-2-yl)-2- 5-diphenyl tetrazolium bromide (MTT) assay. 24×10^4 ADMSCs were seeded in a 96-well culture plate. After reaching the appropriate confluence, the medium with different concentrations of treatment was added to each well, and at 24 and 72 h intervals, the medium was removed from the wells and then solution containing 10% MTT was added. After 3 h of incubation, DMSO was added to the culture plate and OD was measured at 570 nm.

2.5 Cell migration assay

To perform this assay, 60×10^4 ADMSCs were seeded in a 24 well culture plate. After reaching the appropriate confluence, a scratch was created with a 100 µL tip on each well. Then the medium was removed and each well was washed with PBS. Subsequently, treatments with different concentrations were added to each well. An image was taken of each well at intervals of 0, 6, 18, 24, 48, 72, 96, and, 120 h with an invert microscope. The area of each scratch at interval times was obtained using (NIH) Image J software. Wound closure percentage was evaluated using the equation below:

$$\text{Wound closure } \% = \text{AT } h_0\text{-AT } h_n/\text{AT } h_0 \times 100 \quad (2)$$

Where AT h_0 is the area of the wound immediately after scratching;
AT h_n is the area of the wound measured n hours after scratch.

2.6 Statistical analyses

Cell viability and cell migration data were shown as means ± standard deviation. Each treatment had 3 replicates. Statistical analyses were performed using the SPSS statistical package. Analysis of variance (ANOVA) was followed by the Dunnett test in the comparison of the means of MTT assay, and scratch assay means.

3. Results and Discussion

3.1 Morphology of PPE-SA from scanning electron microscopy (SEM)

As can be seen in Figure 1, the PPE-SA formed irregular particles and flake-like structures with spherical morphology and good polydispersity. The particle size was between 10 and 50 nm, which indicated that the resulting polymeric polysaccharide (fucoidan) had a suitable molecular weight, which had an important effect on its biological properties. It has been documented that the extraction method may affect degradation of polysaccharide molecules and thus result in a significant decrease in the particle size of nanocrystal units, decreasing the pharmacological activities of the final products [13, 14].

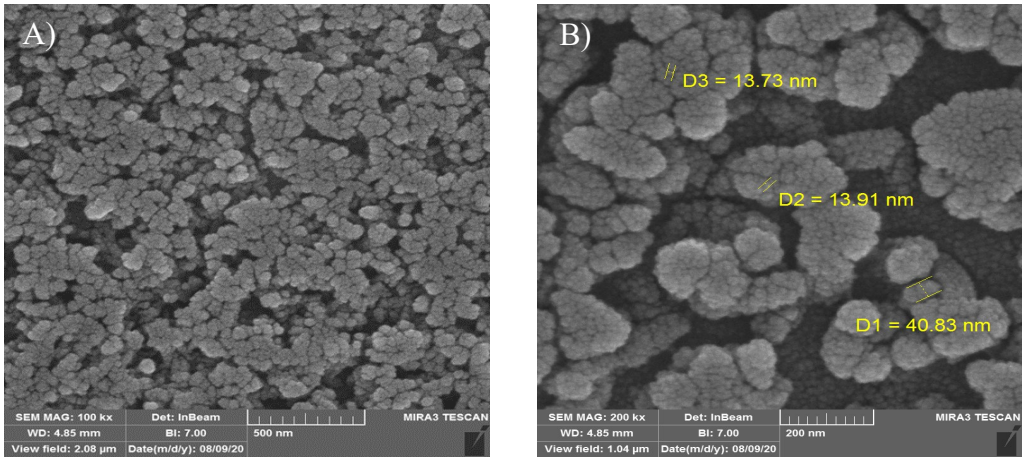

Figure 1. SEM micrograph of PPE-SA

3.2 FTIR spectra of extract

The FTIR spectra of PPE-SA can be seen in Figure 2. Polysaccharides mostly have common peaks with two bands at the 2000-4000 cm^{-1} range and numerous similar peaks in the range of 800-2000 cm^{-1}. Symmetrical and asymmetrical stretching vibrations of O-H were assigned to the broad band at 3426 cm^{-1}. Peaks at 2923 cm^{-1} were due to C-H stretching vibrations. The absorption band at 1623 cm^{-1} indicates H-O-H stretching vibration and moisture. Since *S. angustifolium* is rich in fucoidan, and this polysaccharide is often composed of fucose (a monosaccharide containing the methyl group in the C-5 position), typical bands of PPE-SA correspond to sulfate and methyl group bonding [1, 11]. The peak at 1427 cm^{-1} was assigned to the asymmetrical bending vibration of CH_3 [15]. Because of C-C, C-O stretching in pyranoid rings and C-O-C stretching in glycosidic bonds, we observed IR absorption bands at 1256 cm^{-1} and 1055 cm^{-1}, which are commonly recognized in all polysaccharides [3, 16]. Additionally, the peak at 965 cm^{-1} indicates asymmetrical stretching vibration of C-O-S bonds [11, 17]. The band at 833 cm^{-1} was assigned to the presence of sulfate at the C-4 position [11]. Therefore, due to the presence of characteristic bonds of fucoidan including sulfate, methyl groups, and C-O-S bonds in the polysaccharide extracted from *S. angustifolium*, it was concluded that PPE-SA had an abundance of fucoidan which was consistent with the L-fucose assay.

3.3 ICP-OES analysis

As shown in Table 1, PPE-SA had an element concentration in this order: Ca>K>S>Na>Mn>P>Sr>As>Cu>Mo>Zn=B>Ti>Pb>Cd. High levels of calcium ions (98.71 PPM) in the extract can play a role in bone regeneration. Studies have also shown that calcium ions have been significantly effective in the migration and proliferation of mesenchymal stem cells [18]. It may be hypothesized that the high level of Ca was caused by the addition of $CaCl_2$ to remove alginate during sample preparation. It must be mentioned that in the extraction process, calcium chloride can combine with alginate and produce an alginate/$CaCl_2$ precipitation [19]. However, after two centrifugation steps at 11000 rpm, we discarded the sediment and used the supernatant. Therefore, the possibility of the presence of calcium chloride and the subsequent increase of calcium levels in the extract was very low.

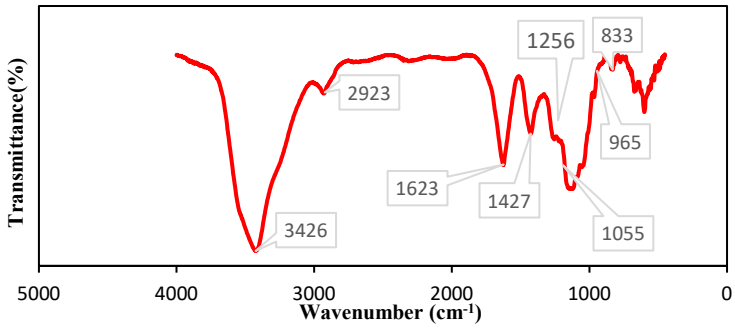

Figure 2. FTIR spectra of PPE-SA

Table 1. Analysis of PPE-SA by ICP-OES

Elements														
K	Ca	S	P	Mn	Mo	B	Zn	Cu	Na	Cd	Pb	Sr	Ti	As
Concentration (ppm)														
61.5	98.7	35.4	1.5	6.7	0.66	0.2	0.20	0.98	20.8	<0.02	0.15	1.10	0.17	1.00

According to the information available on the official website of the European Commission [20] and the National Health and Nutrition Examination Survey [21], the safety levels of lead and cadmium are 3.0 ppm and 1.0 ppm in food supplements, respectively. The amount of these heavy metals in the extract was lower than the permissible limit. However, the amount of arsenic in the extract was higher than the standard level [20, 21]. Therefore, researchers should consider this point when conducting further research on *S. angustifolium*.

The high concentration of sulfur in the extract was due to the presence of fucoidan polysaccharide, which is consistent with the FTIR results and L-fucose assay.

3.4 CHNS analysis

The elemental composition is shown in Table 2. The presence of nitrogen (N%) is due to the existence of a few amino-containing compounds such as protein and amino sugars. The relationship between fucoidan bioactivity and its sulfur content previously attracted the attention of researchers. Most of these studies were focused on the anticoagulant activity of fucoidan. The results showed that sulfur-rich fragments with low uronic acid content had more anticoagulant activity than vice versa [22, 23]. Therefore, it can be concluded that the extraction methods preserve the biological properties of fucoidan.

Table 2. CHNS analysis of PPE-SA

C	H	N	S	DS
		%		
24.39	5.56	0.19	6.05	0.55

3.5 L- fucose assay

Fucose is the main monomer of fucoidan; therefore, its content was calculated in the brown algae extract by the colorimetric method. The percentage of fucose in the extract was 35.08%. This value was in the range published in many studies, and is responsible for the antioxidant and mobilization properties of fucoidan [3, 24, 25]

3.6 Cell proliferation

The non-toxicity of the extract is essential for biological applications. For this reason, the cell viability of ADMSCs was assessed at 24 and 72 h by MTT assay (Figure 3). No cytotoxicity was observed between the different concentrations of PPE-SA extract. Most of the treatment groups increased the cell viability significantly in comparison to the control group. At the concentrations studied, PPE-SA did not only reduce the cell viability compared with the control, but also promoted cell growth, all of which pointed to the great biocompatibility of the material.

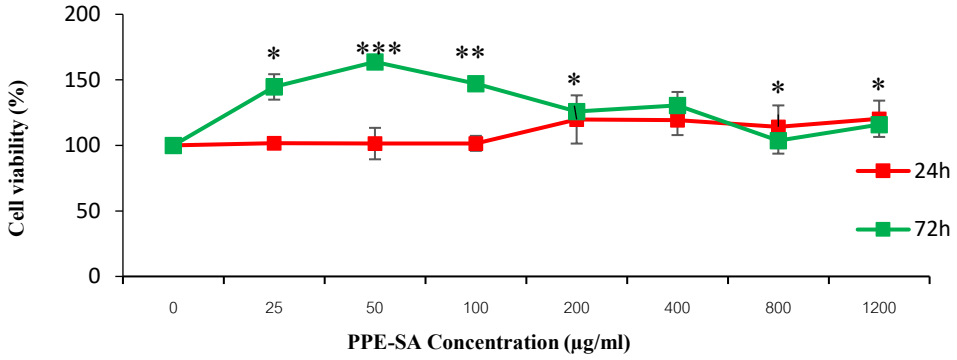

Figure 3. Cell viability of adipose derived mesenchymal stem cells at different concentration of *S. angustifolium* extract at 24 h and 72 h after incubation. Values were mean ± SD (n = 3), *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$ compared with the control^{+} (zero concentration) in the same group using Dunnett's test.

3.7 Scratch assay

Adipose derived mesenchymal stem cells (ADMSCs) are able to differentiate into different lineages and to secrete paracrine factors initiating tissue regeneration process. It has been postulated that ADMSCs can initiate or enhance tissue regeneration by two different mechanisms, either by differentiating into skin cells or by secretion of paracrine factors which can initiate the healing process via recruitment of endogenous stem cells and endothelial cells or by down-regulating the inflammatory response [26]. Skin wounds treated with ADMSCs have shown enhanced healing via epithelial migration, angiogenesis with better healing rate, and less scar formation [27]. According to these documents, we investigated the effect of *S. angustifolium* extract on the proliferation and migration of ADMSCs, which are directly effective in wound healing.

According to the results of the MTT assay, the concentrations of PPE-SA which had positive and significant effect on cell growth in 24 h incubation were selected for the scratch assay. Therefore, the effect of the lowest and highest concentrations with this feature, i.e. 200 µg/mL and 1200 µg/mL were evaluated on ADMSCs migration by scratch assay. In this experiment, to reduce

the effect of proliferation on the results, before and after scratching, cells were treated with 5% FBS. A well with no treatment was considered as negative control and after scratching, 10% FBS was added to the positive control well plate. Figure 4 displays percentage of wound closure of ADMSCs. It was observed that *S. angustifolium* extract significantly improved wound closure as compared to the positive and negative controls, indicating its positive effect on cell migration by as much as 76% and 142% at 200 µg/mL after 48 and 72 h incubation, respectively. Bouvard *et al.* [7] reported that pure fucoidan extracted from the brown algae *Ascophyllum nodosum* induced endothelial cell migration by 40% at 10 µg/mL after 24 h incubation, via the PI3K/AKT pathway and modulated the transcription of genes involved in angiogenesis, while its effect on blood monocyte adhesion and migration was 45% compare with that of control [7]. Lake *et al*. [5] also found that pure fucoidan extracted from brown seaweed at 10 µg/mL increased endothelial cell migration by 50% at 10 µg/mL after 20 h incubation, via enhancement of vascular endothelial growth, while SDF-1 as an important chemokine in stem cell mobilization promoted the migration of this stem cell only by 30% [6]. Kim *et al.* [15] reported a 40% positive effect of pure fucoidan extracted from *Undaria pinnatifida* on osteoblast cell migration at 50 µg/mL after 24 h incubation, but it effects at concentration of 200 µg/mL were not significant compared with that of control. It also had cytotoxic effect at 400 µg/mL [15]. It was observed that all above mentioned experiments used pure fucoidan; therefore, at low concentrations and over short incubation times, they had a significant effect. But as expected, the effect of the crude extract of most seaweeds such as in the current study, has been significant only at high concentrations and over longer incubation times. Cui *et al.* [28] found that crude fucoidan extracted from *Stichopus japonicus* promoted neural stem cell migration by only 30% at 80 µg/mL after 24 h incubation. This effect for pure fucoidan was 100% at 10 µg/mL [28]. Premarathna *et al.* [29] studied the cytotoxicity and wound healing activity of aqueous extracts of 22 seaweed species on mouse fibroblast (L929) cells. The seaweed samples belonged to *Phaeophyta* (brown), *Chlorophyta* (green) and *Rhodophyta* (red). In this experiment, contrary to our method, impurities in the extracts, including pigments, lipids, and low molecular weight compounds were not removed with ethanol, and alginate was not removed by $CaCl_2$. In a very simple way, these 22 types of seaweed were extracted with distilled water. Seaweed aqueous extracts were tested for cytotoxic activity and wound healing. The results showed that the highest migration effect belonged to the green seaweed *Halimeda opuntin* and an extract of a brown seaweed, *Stoechospermum polypodioides*. They were able to improve wound healing by 100%, but at a very high concentration of about 4000 µg/mL and an incubation time of 24 h. It is interesting to note that out of the 22 species, only 8 cases had a positive effect, while the other 16 species were either ineffective or even had a negative effect [29]. In the current study, *S. angustifolium* increased cell migration of ADMSCs by 76% and 142% at concentration of 200 µg/mL at 48 and 72 h incubations (Figure 4).

The images obtained with the invert microscope at different time intervals were also shown the migration effect of PPE-SA on ADMSCs in comparison to the negative and positive control groups (Figure 5 and Figure 6). These results showed that water/ethanol crude extract obtained by Borazjani *et al.* [3] (used in the current study) was completely superior to aqueous extract obtained by Premarathna *et al.* [10]. As found by other researchers [5, 15], the results show that increasing the concentration of fucoidan from 200 to 1200 µg/mL decreased its effect.

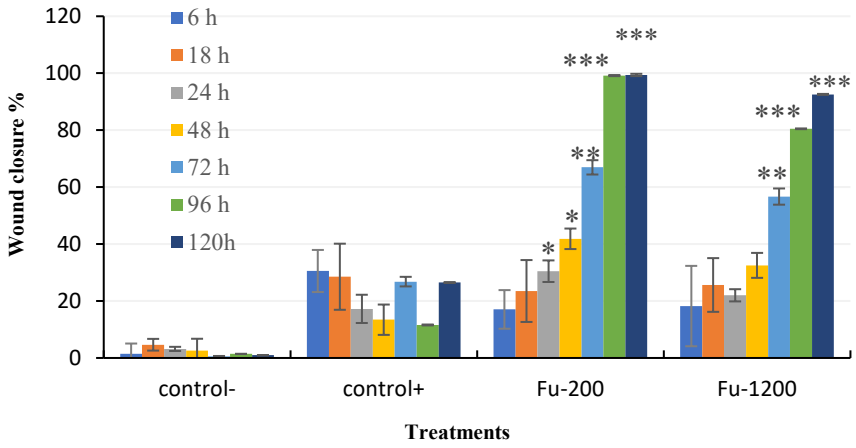

Figure 4. Scratch assay of adipose derived mesenchymal stem cells at different concentration of *S. angustifolium* extract. Values were mean±SD (n =3), *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$ compared with the control^{+} (zero concentration) in the same group using Dunnett's test.

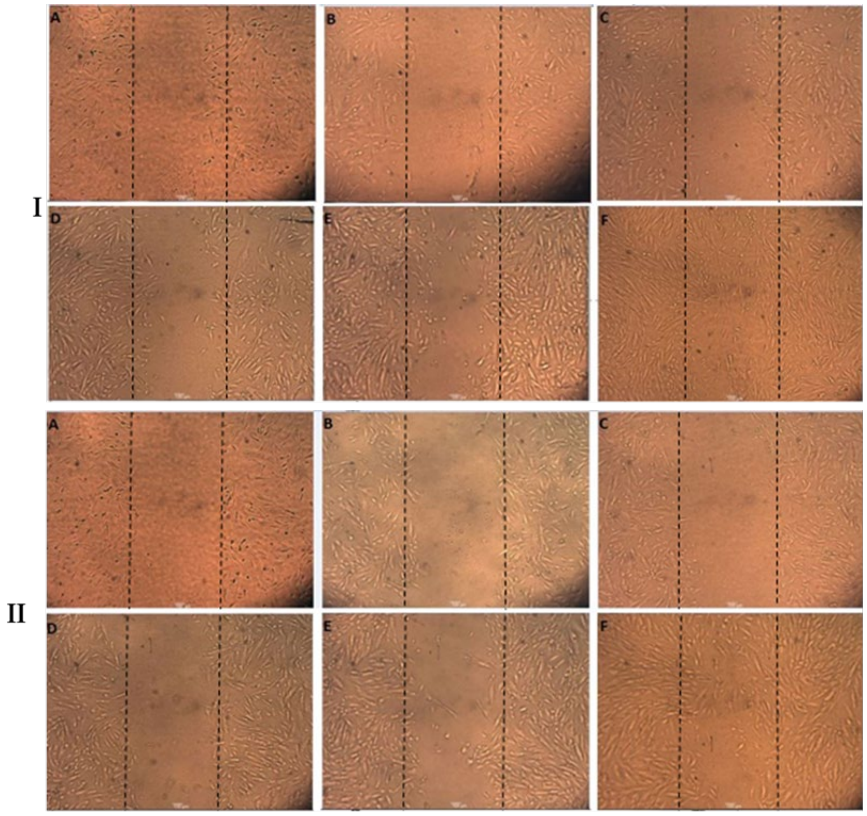

Figure 5. Scratch assay of adipose derived mesenchymal stem cells in I (PPE-SA at 200 µg/mL) and II (PPE-SA at 1200 µg/mL) treatments at 0 h (A), 6 h (B), 18 h (C), 24 h (D), 48 h (E),72 h (F) after scratch

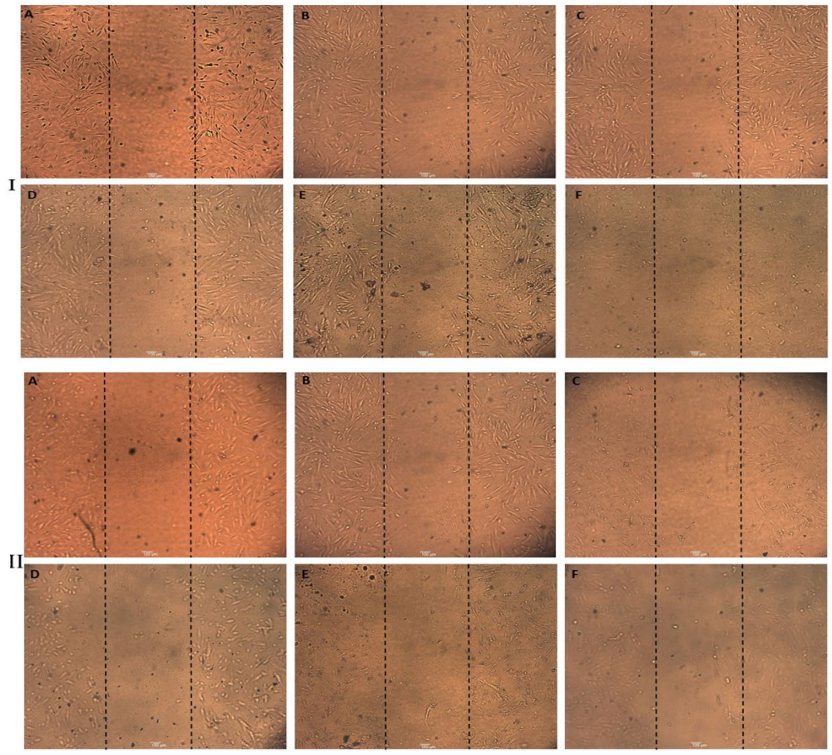

Figure 6. Scratch assay of adipose derived mesenchymal stem cells in I (positive control) and II (negative control) treatments at 0 h (A), 6 h (B), 18 h (C), 24 h (D), 48 h (E),72 h (F) after scratch

4. Conclusions

In this study, crude fucoidan (PPE-SA) of *S. angustifolium* was successfully extracted. The effect of PPE-SA on the proliferation and migration of stem cells were measured. In cellular experiments, PPE-SA did not exhibit cytotoxicity within 72 h even at high concentrations, which indicated the safety of the substance. The scratch assay revealed that the PPE-SA significantly contributed to the migration of ADMSCs, a result which suggested its potential use in wound healing.

5. Acknowledgements

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