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Article

Evaluation of the Pro-Angiogenic Effect in Chicken Embryo Chorioallantoic Membrane (CAM) Model and the In Vivo Wound Healing Potential of the Moroccan Polysaccharide-Rich Algae *Osmundea pinnatifida*

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Abstract: Various methods have been studied to speed up the wound healing process. Promoting the rapid development of blood vessels, also known as angiogenesis, is essential as this significantly accelerates skin regeneration. This study identified a polysaccharide from the red alga *Osmundea pinnatifida* (PSOP) that promotes angiogenesis and accelerates wound healing. Firstly, we analyzed the structural characteristics of PSOP using various techniques such as UV-visible spectrum, Fourier-transform infrared (FT-IR) spectroscopy, X-ray diffraction (X-RD), scanning electron microscopy (SEM), and high-performance liquid chromatography coupled to refractive index detector (HPLC-RID). We also evaluated the antioxidant properties of PSOP in vitro using tests for DPPH free radical scavenging capacity, iron reduction capacity (RPA), and total antioxidant capacity (TAC). Additionally, we observed a significant increase in new blood vessel formation in the chorioallantoic membrane model (CAM assay) using a fertilized hen egg, indicating the pro-angiogenic effect of PSOP. Furthermore, we tested the wound-healing potential of PSOP hydrogel *in vivo* using excision wounds in the Wistar rat model. The results showed a notable acceleration of wound closure and an improvement in tissue regeneration and collagen deposition quality within 11 days, indicating the strong wound-healing activity of PSOP. Our computational study suggested that the PSOP units bind to cyclooxygenase 2 (COX-2) and vascular endothelial growth factor (VEGF) with acceptable affinities and interactions, which might explain the observed beneficial wound healing properties and the pro-angiogenic effect seen in the experimental assays. Our findings support using the polysaccharide PSOP for wound treatment due to its antioxidant and pro-angiogenic potential.

Keywords: *Osmundea pinnatifida*; polysaccharide; wound healing; angiogenesis; CAM assay; molecular interactions

1. Introduction

Wounds, whether caused by surgery, burns, cuts, or severe traumatic lacerations, require effective healing. Rapid restoration of microvascular connectivity is essential to reduce ischemia and hypoxia, facilitating tissue repair and restoration processes [1]. In general, acute wound healing can take up to four weeks and involves hemostasis, inflammation, proliferation, and remodeling [2]. Furthermore, wounds are caused by a disturbance in angiogenesis, one of the leading causes of delayed wound healing. Wound healing is promoted by improved angiogenesis, which reduces excessive inflammation and provides damaged tissue with the oxygen and nutrients essential for regeneration [3]. Disturbances at any of these stages can lead to the formation of chronic wounds, such as pressure sores, diabetic ulcers, and vascular wounds. These wounds increase treatment costs and are characterized by persistent inflammation, resistance to therapies, and insensitivity of cells to healing signals, ultimately leading to a significant increase in morbidity and mortality [4], [5].

Researchers have been studying bioactive compounds from marine algae for their potential therapeutic uses in various diseases. Algal polysaccharides, in particular, have drawn significant interest due to their diverse biological activities, including anti-inflammatory, anticoagulant, antitumor, antiviral, and antioxidant properties [6], [7], [8], [9]. In addition, it has adhesive properties, is biodegradable, non-toxic, and has various physical and chemical characteristics [10]. Lately, inexpensive natural polysaccharides used in skin therapeutics have attracted attention for their effectiveness in wound healing [11] by promoting cell regeneration and collagen synthesis.

Osmundea pinnatifida, synonym *Laurencia pinnatifida* (Rhodobionta, Rhodophyta), is a red macroalgae, widespread (Class: Florideophyceae; Order: Ceramiales; Family: Rhodomelaceae). It's edible for human food and dietary supplements because it contains vitamins, proteins, amino acids, fiber, and fatty acids [12]. Therefore, it represents an essential source of agar production [13], which can be investigated in the food fields. A present review highlights the biotechnological potential of these algae, including their antitumoral, antiviral, antiprotozoal, antibacterial, and antifungal activities [14]. Also, previous studies have revealed that this alga's aqueous and hydro ethanol extract presents an antioxidant and anti-aging activity [15]. An enzymatic extract of *O.pinnatifida* has demonstrated important biological properties, such as antioxidant and anti-diabetic [16]. However, polysaccharide extracts from this alga remain primarily under-exploited, and their biological applications have yet to be thoroughly investigated.

The current study was designed to extract a polysaccharide from the red alga *Osmundea pinnatifida*, named PSOP. Firstly, the structural characteristics of the polysaccharide have been investigated. The characterization of PSOP was determined using chemical and instrumental analysis, high-performance liquid chromatography (HPLC-RID), UV-visible spectrum, FT-IR spectroscopy, X-ray diffraction (X-RD), and scanning electron microscopy (SEM). Molecular weight, total sugars, sulfates, and proteins were then determined. The *in vitro* antioxidant activities of PSOP were assessed by a DPPH Free radical assay, as well as total antioxidant activity and reducing power. In the second part, the pro-angiogenic potential of PSOP has been demonstrated *in ovo* via chicken embryo chorioallantoic membrane (CAM) and validated *in vivo* by accelerating the wound healing process using an excision model in Wistar rats. Additionally, the PSOP units were further explored using a computational approach, mainly by assessing binding affinities and molecular interactions by targeting cyclooxygenase 2 (COX-2) and vascular endothelial growth factor (VEGF).

2. Material and Methods

2.1. Algae Collection and Identification

Osmundea pinnatifida was collected from the Atlantic coasts of Sidi Bouzid (El Jadida, Morocco; 33°13'N-8°55'W) (**Figure 1**) between March and May 2023. The algae were identified at the laboratory of Marine Biotechnology and Environment, Faculty of Science of Chouaib Doukkali University. They were carefully washed, shade dried, milled, and stored in amber glass bottles at 4°C.

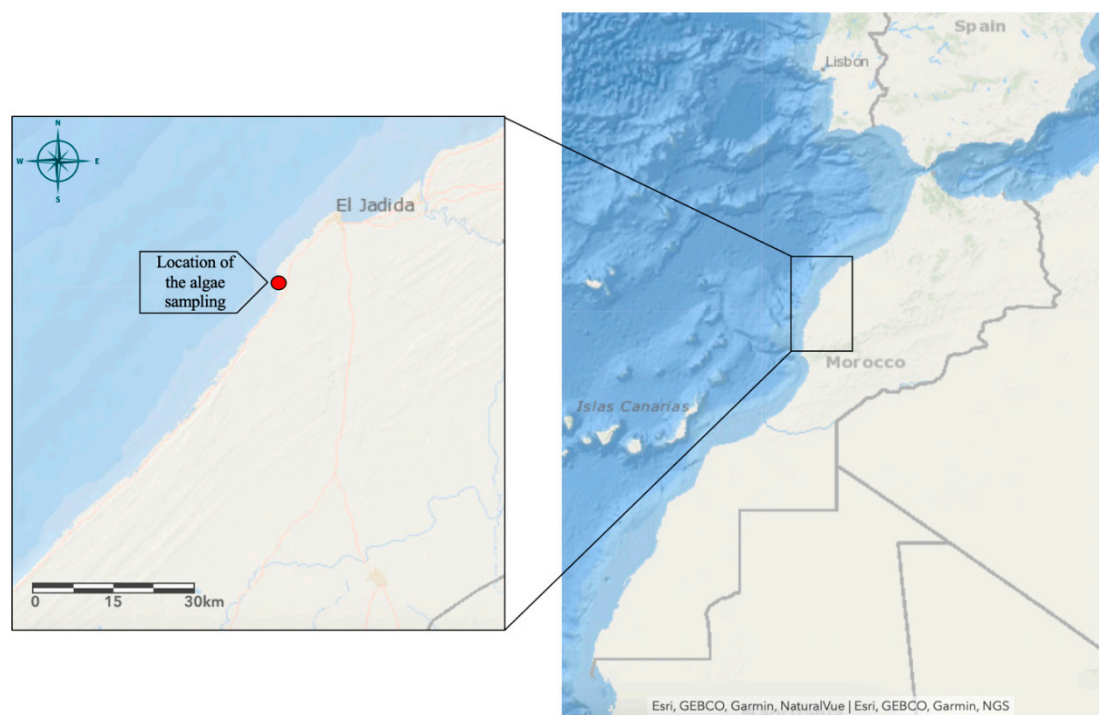


Figure 1. The location of the seaweed collection site uses ArcGIS version 10.7 (Sidi Bouzid coastal zone, El Jadida, Morocco).

2.2. Extraction of the Polysaccharide-Rich *Osmundea pinnatifida* (PSOP)

Red algae pigments were dissolved in 95 % ethanol before the PSOP extraction, according to Lui et al. [17]. One hundred grams of dried seaweed powder were stirred for 4 hours with distilled water at 90 °C. The crude water extract was filtered, vacuum dried, and precipitated for 24 hours at 4 °C in 95 % ethanol (3v/v). The insoluble polysaccharide-rich was pelleted by centrifugation (6000 rpm, 20 min, 4 °C) using a refrigerated benchtop centrifuge (Hettich, Rotina 380R). For three days, PSOP was resuspended in distilled water and lyophilized (Christ Alpha 1-2 lyophilizer, Bioblock Scientific). The resulting extract, PSOP, was stored at 4 °C until use.

2.3. Chemical Characterization

The yield was expressed in the ratio of the dry weight of the polysaccharide extracted (g) against the dry weight of *Osmundea pinnatifida* (g) in percentage. Total carbohydrate content was determined using the phenol-sulfuric acid colorimetric method [18], protein concentration was determined using the method of Lowry et al. [19], and a turbidimetric method was used to quantify the sulfate content using the barium chloride/gelatin reagent [20].

2.4. Polysaccharides Spectroscopic Analysis

2.4.1. Ultraviolet Absorption Peak Detection

The polysaccharide was prepared in distilled water to a final concentration of 1 % (w/v). The solution's UV-vis absorption spectrum was registered in the 200-800 nm wavelength range using a UV-vis spectrophotometer (JENWAY/7315, UK) [21].

2.4.2. Fourier Transform Infrared Spectrometry Analysis

The PSOP powder was applied to a Nicolet FT-IR spectrometer fitted with an attenuated reflection attachment (ATR) containing a diamond/ZnSe crystal. The spectrum was acquired with a resolution of 4 cm⁻¹, and the measurement range was 400 to 4000 cm⁻¹ at room temperature (25 °C). Data analysis and processing were carried out using OPUS (Bruker, Ettlingen, Germany) [22].

2.4.3. Monosaccharide Analysis by High-Performance Liquid Chromatography (HPLC-RID)

Monosaccharide compositions were analyzed using the method [23]. A 5 mg sample was dissolved in 3 mL TFA (2 mol/L) and hydrolyzed at 110 °C for 3h. The TFA was removed by washing with methanol. The hydrolyzed product was then reduced by adding 50 mg sodium tetrahydruborate. Pyridine and acetic acid anhydride were added to the mixture at 40 °C for 2 h for acetylation. The acetylated sample was filtered and examined using high-performance liquid chromatography (LC1260 Infinity II), equipped with an Aminex HPX-87C column with H₂SO₄ at (0.001 N) as mobile phase, programmed with a flow rate of 0.4 mL/min and a column temperature of 60 °C. The standard sugars utilized were glucose, fructose, xylose, mannose, and glucuronic acid.

2.4.4. The Average Molecular Weight

According to Bayar et al. [24], the average molecular weight (Mw) was determined by steric exclusion chromatography (SEC).

2.4.5. Scanning Electron Microscopy

PSOP surface mapping, microstructure assessment, and morphology analysis were investigated using cryogenic scanning electron microscopy (JSM-IT500HR, JEOL).

2.4.6. X-Ray Diffraction

The XRD diffractogram of PSOP was performed using an X-ray diffractometer (D2 PHASER, Bruker, German, Berlin) at room temperature. The measurement was conducted in the 2 ranges from 10 to 80 ° (2θ) with a step size of 0.02 ° and a counting time of 5 s/step.

2.5. Antioxidant Activity of Polysaccharide

2.5.1. DPPH Free Radical Scavenging Assay

DPPH radical scavenging activity was measured according to [25]. PSOP at different concentrations (1-10 mg/mL) was incubated with 0.02 % DPPH (in ethanol). DPPH radical reduction was measured at 517 nm. The standard used was butylated hydroxyanisole (BHA). DPPH radical scavenging capacity was calculated with the following equation:

$$\% \text{ DPPH scavenging} = \left[\frac{AC + AS - AB}{AC} \right] \times 100$$

where AC, AB, and AS refer to the optical densities of blank, control, and sample, respectively.

2.5.2. Total Antioxidant Activity

Total PSOP antioxidant activity was assessed according to the protocol in [26]. Absorbance was measured at 695 nm against a blank. The standard used was butylated hydroxyanisole (BHA).

2.5.3. Reducing Power Assay

The ability of PSOP to reduce iron was determined according to the method of [27]. The absorbance of the resulting solution was measured at 700 nm. Butylated hydroxyanisole (BHA) was used as the standard.

2.6. Chorioallantoic Membrane Assay

Angiogenesis activity was assessed *in ovo* using the chorioallantoic membrane (CAM) test with some modifications [28]. The experiment presented in the study does not require approval from the ethics committee [29].

In summary, fertilized chicken eggs from the Eurafic Poussins Company in Had Soualem, Morocco, were first cleaned with 10% betadine and then placed in an incubator at 37°C with 55-65% humidity for 3 days. The eggs were manually rotated three times a day to ensure proper development of the chicks. On the fourth day, 3 mL of albumin was removed using a sterile syringe with a 20-gauge needle, and the incubation process continued. On the eighth day, a small opening was created on the larger side of the egg, and a 1 cm² window was carefully made under a laminar flow hood. The chorioallantoic membrane was then removed without causing harm to the blood vessels. Then, the eggs were divided into 4 experimental groups (n = 6/treatment):

Group 1: eggs were treated with sterile distilled water (the control);

Group 2: eggs were treated with diclofenac DIC (50 µg/egg), which was used as a negative control;

Group 3: eggs were treated with choriogonadotropin CG (30 µg/egg), which was used as a positive control;

Group 4, 5, and 6: eggs were treated with various concentrations (25, 50, and 100 µg/egg) of PSOP;

The eggs were re-incubated for three days after sealing the windows with transparent adhesive tape. On day 12, the neovascular regions of the CAM were photographed using a stereomicroscope (Steroblue, EUROMEX).

2.7. Quantitative Analysis of Vascular Network

The photomicrographs obtained from the CAM vessels were analyzed using ImageJ software (version 1.54, National Institutes of Health, USA) and AngioTool software (version 0.6a, National Cancer Institute, USA). To quantify the topography of the vascular network, using the software ImageJ, areas of 500 mm² were selected; the images were converted to grayscale and, in sequence, "inverted" from the plugin "edit." After that, the background was extracted from the photos (a value of 50 pixels was fixed) in the "process" plugin. The photomicrographs were saved and then opened using the AngioTool software. A typical image displaying the network topology was detected, formed by vessel area, vessel length, vessel diameter, number of junctions, and lacunarity (avascular space).

2.8. In Vivo Experimental Study

2.8.1. Animals

Twenty-four healthy adult male Wistar rats weighing 150-200 g were obtained from the Pasteur Institute of Tunis, Tunisia. The rats were kept in a ventilated room, in individual cages at room temperature (22-24 °C), with a 12-hour light and 12-hour dark cycle and free access to food and water.

2.8.2. Experimental Protocol

The excision model was used to assess the extent of wound contraction. Twenty-four rats were divided into four control and test groups:

Group 1: was treated with saline (0.9 %) and used as a control group.

Group 2: was treated with a standard drug, "Cytol Centella" cream, used as a medicated reference cream.

Group 3: was treated with PSOP hydrogel (lyophilized PSOP was mixed in 30% glycerol to obtain a final concentration of 15 mg/mL).

Group 4: was treated with glycerol (30 %).

After cleaning the rats' skin with sterile water, it was disinfected with swabs soaked in 70 % alcohol. A light shave was performed on the back of the interscapular skin area. In brief, a circular wound of approximately 150 mm² (1.5 cm in diameter) was made using a sharp surgical blade. All wounds were cleaned and treated every two days with sterile saline, vehicle, PSOP, and the reference drug "Cytol Centella" in a thin layer covering the wound surface until the wound was completely healed. On day 12, after wounding, all rats were anesthetized with ether and sacrificed by decapitation. Part of the re-epithelialized tissue was preserved for hydroxyproline estimation, and another part was fixed in 10 % (v/v) formalin, embedded in kerosene, and processed for histological observation.

2.8.3. Measurement of Wound Area and Burn Contraction Rate

Photographs were taken of the wound surface on days 1, 5, 7, and 11 for evaluating the healing progress. The wound surface was measured using Autodesk AutoCAD software. The rate of wound contraction can be calculated using the equation below:

$$\text{Rate of wound contraction(\%)} = \frac{\text{initial surface size} - \text{specific day surface size}}{\text{initial surface size}} \times 100$$

2.8.4. Determination of Hydroxyproline Content

Hydroxyproline concentrations were calculated using the method of [30]. In brief, the wound biopsies were dried at 60 °C until constant weight was obtained. Then, they were hydrolyzed with 6N HCl for 3h at 120 °C. The hydrolyzed specimens were subjected to chloramines-T-oxidation and stored at room temperature for 20 min. The color developed with the presence of Ehrlich's reagent (2.5 g of 4-dimethylamino benzaldehyde, 9.3 mL of n-propanol, and 3.9 mL of 70 % perchloric acid) at 60 °C was measured at 557 nm after cooling. The results were reported as mg/g dry weight of tissue.

2.8.5. Histological Examination

On the day one of the groups completely healed, the rats were sacrificed, and the wound site biopsies were collected and fixed immediately in formalin solution (10 %). They were then embedded in kerosene, stained with hematoxylin-eosin, and examined with a light microscope (Olympus CX41, Tokyo, Japan).

2.8.6. Statistical Analysis

The results obtained were expressed as means ± standard deviation and were analyzed with SPSS using analysis of variance (ANOVA). When the ANOVA revealed a significant effect, the Tukey test post hoc compared the values. Differences were considered statistically significant at $p < 0.05$.

2.9. Computational Modeling and Interactions Assay

In an attempt to rationalize the potential effect of the pro-angiogenic and wound healing of the identified *O. pinnatifida* monosaccharides, a computational modeling and interactions assay was realized. In this context, the binding affinity of all the identified phytochemicals with some key

macromolecules involved in both pro-angiogenic and wound healing have been assessed. The crystal structures of cyclooxygenase 2 (COX-2, pdb id: 1cx2) and vascular endothelial growth factor (VEGF, pdb id: 2c7w) have been collected from RCSB databases. These macromolecules have been prepared by the deletion of crystalized water molecules and the addition of both polar hydrogen and Kollman charges... before being processed for CHARMM force field as previously described ([31], [32], [33]). The established bond categories and molecular interactions have been recorded and analyzed ([34], [35], [36]).

3. Results and Discussion

3.1. Chemical Characterization of PSOP

In this study, the yield of PSOP was estimated to be 32.65 %. According to Arunkuma et al. [37], crude polysaccharides isolated from *L. papillosa* and *L. obtusa* yielded around 4 % and 6 %, respectively. Polysaccharide yields vary according to species, environmental conditions, and extraction methods [38], [39]. The total sugar content of PSOP was about 88.12 % $\pm$ 1.1 %, which was higher than what was reported for algae *L. pedicularioides* and *L. cruciata* (44.8 % and 49.4 %, respectively) [40]. The results of various colorimetric tests showed that the polysaccharide analyzed contained low levels of protein (0.18 % $\pm$ 0.02 %) and low levels of sulfate (0.69 %), proving the extraction method's precision. The polysaccharide obtained by ethanol precipitation frequently contains contaminants, such as proteins, which require purification by deproteinization [41]. However, this process can alter carbohydrate yields, modify monosaccharide composition, influence structural parameters, and affect polysaccharide bioactivity [42], [43]. Following on from the proteins discussed above, it's worth pointing out that sulfate groups linked to polysaccharides also influence crucial biological functions, such as antitumor, anticoagulant, and immunomodulatory effects [44].

3.2. Spectroscopic Analysis and Monosaccharide Composition

Figure 2A illustrates the UV-visible spectra of PSOP. Our finding revealed a maximum absorption peak between 200 and 240 nm, which confirms that our sample is a polysaccharide [45]. A weak peak was observed at 260-280 nm, which indicates the presence of the protein trace or nucleic acid covalently linked to PSOP [46]. An absorption area identified at around 300 nm was attributed to trace pigments.

As is well known, FT-IR spectroscopy can be used to identify structural features of polymer blends, such as distinct organic groups in the polysaccharide. **Figure 2B** shows that PSOP exhibited absorption peaks typical of polysaccharides between 700 and 4000 cm^{-1} . A broad hydroxyl group O-H stretching vibration peak was recorded at around 3389 cm^{-1} due to intra- and intermolecular hydrogen bonds. Subsequently, a weak peak detected at about 2929 cm^{-1} was attributed to the C-H stretching vibration of sugars [47], indicating that the sample was a polysaccharide compound. Peaks appearing at about 1637 and 1412 cm^{-1} , respectively, were attributed to carboxylate bond stretching, demonstrating that PSOP was an acidic polysaccharide [48]; this suggests the presence of uronic acids, an observation corroborated by analysis of monosaccharide composition. The hydroxyl and carboxyl groups of polysaccharides are essential to their biological properties, notably to enhance their antitumor and antioxidant effects [49]. In addition, the weak absorbance peak at 1369 cm^{-1} was attributed to ester sulfate S=O groups [50]. Absorption peaks between 1136 and 1047 cm^{-1} were associated with C-O-C and C-O-H stretching vibrations, characteristic of the pyranose ring structure [51]. Combined, weak absorptions around 800 and 900 cm^{-1} originate from the stretching vibration of the C-O-S [52], indicating the existence of α and β configurations in the polysaccharide.

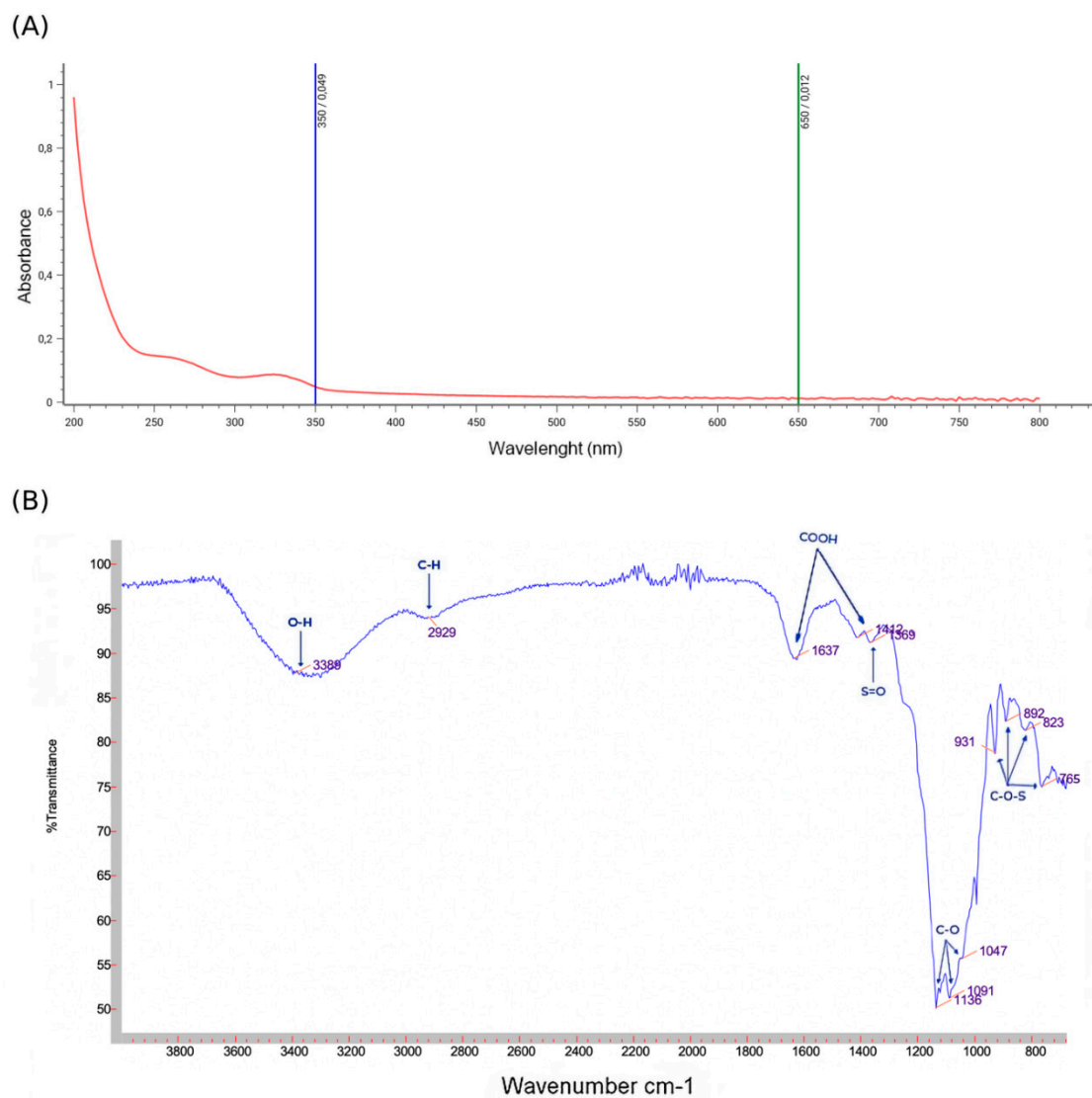


Figure 2. Preliminary characterization of PSOP. (A) UV-vis absorption spectrum of the PSOP; (B) Infra-red spectrum of the PSOP.

Figure 3A represents the HPLC-RID chromatogram of the monosaccharides detected in the PSOP. The analysis by high-performance liquid chromatography with a refractive index detector revealed the presence of the different monosaccharides, in particular, glucuronic acid, arabinose, glucose, xylose, and fructose, at retention times of 8.32, 9.39, 11.19, 12.13 and 13.64 min, respectively, by elution time of monosaccharide standards (**Figure 3B**). Further research reveals that red algae polysaccharides are made up of a heterogeneous combination of monosaccharides. For example, the polysaccharide obtained in enzyme extracts from the alga *O. pinnatifida* was composed of galactose, mannose, arabinose, xylose, rhamnose, and fucose [53]. The monosaccharide composition of *Laurencia dendroidal* polysaccharide is mainly galactose, with smaller amounts of xylose, mannose, and glucose [54]. However, several exogenous factors such as protocol, water concentration, extraction temperature, and endogenous factors in the organisms (type of species, reproductive cycle...) can influence the polysaccharide composition [55], [56]. Indeed, the nature and proportion of monosaccharides present represent a key element affecting the activity of polysaccharides, which can confer new bioactive properties or reinforce existing ones [57].

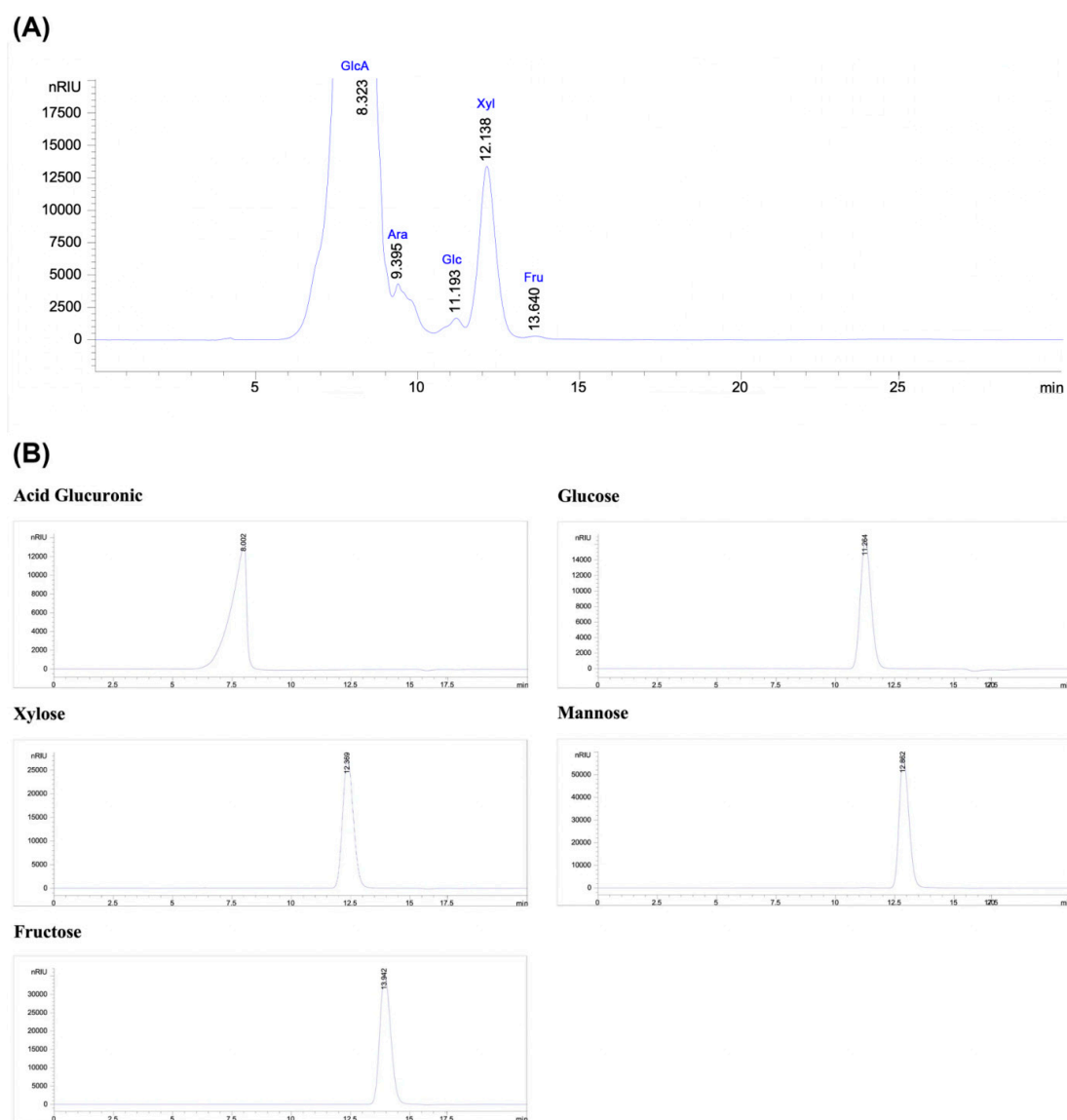


Figure 3. Preliminary characterization of PSOP. (A) Monosaccharide composition analysis by HPLC-RID; (B) standards used for HPLC. GlcA: Acid Glucuronic; Ara: Arabinose; Man: Mannose; Glc: Glucose; Xyl: Xylose and Fru: Fructose.

The molecular weight (Mw) profile was analyzed by steric exclusion chromatography (SEC), revealing that PSOP had a molecular weight of 146 kDa, with a retention time of 7.25 min (**Figure 4**). In reality, the molecular weight of polysaccharides has a considerable influence on their biological activities. In a study by Lee and his collaborators [58], a low-molecular-weight polysaccharide (3 kDa) was obtained by controlled degradation of a high-molecular-weight polysaccharide (2238 kDa). The results showed that the low-molecular-weight polysaccharide possessed superior antioxidant activity and more effectively stimulated the immune response in specific organs subjected to oxidative stress than the higher-molecular-weight polysaccharide.

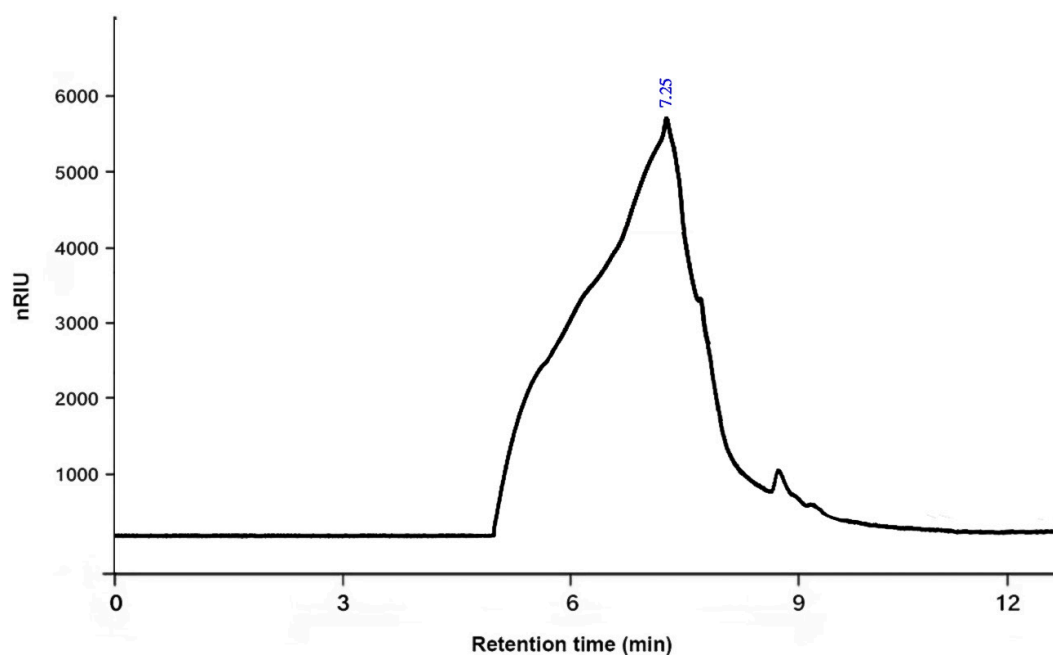


Figure 4. The average molecular weight of PSOP by steric exclusion chromatography (SEC).

Scanning electron microscopy (SEM) is a technique that produces images of the surface of polysaccharides to analyze their structure, including shape, size, and porosity [59]. Scanning electron micrographs of PSOP are illustrated in **Figure 5A**. It was observed that PSOP under 10- and 20-fold magnification had a microstructure in the form of a network with many cavities and had a highly developed porosity over the entire surface area with some heterogeneity, which explains an improved ability to supply oxygen and absorb exudates; a valuable feature from a pharmacological and cosmeceutical point of view [60].

X-ray diffraction is a technique mainly used to identify polysaccharides' crystalline or amorphous structure [61]. **Figure 5B** illustrates that the XRD diffractogram of PSOP, over a diffraction range of 0-80°, displays a broad diffuse band with a dominant peak at around 32° (2θ), accompanied by several other less pronounced peaks. This distribution suggests that PSOP has a semi-crystalline structure in an amorphous matrix. The degree of crystallinity of polysaccharides strongly influences their properties, such as mechanical strength, solubility, and swelling capacity [62]. For example, semi-crystalline to crystalline polysaccharides have improved water retention capacity, making them ideal for applications such as hydrogels, biofilms, nanofibers, membranes, and the medical field [63].

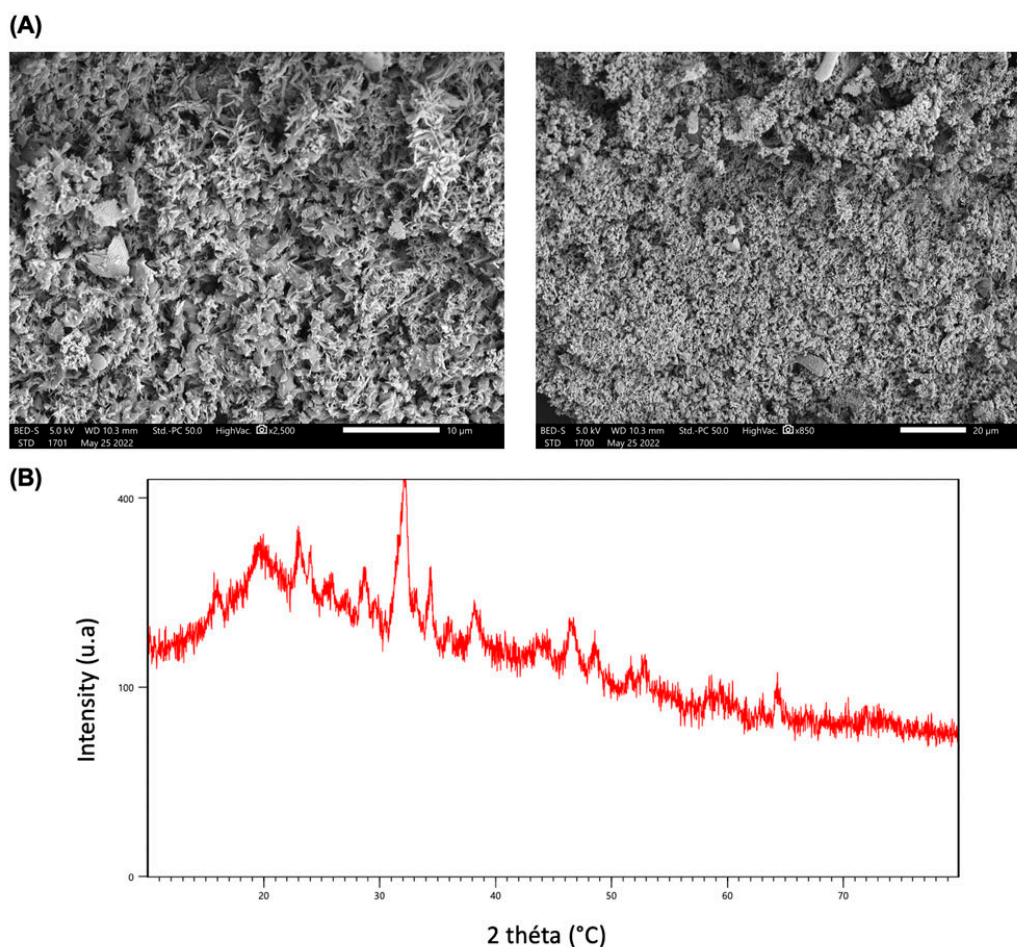


Figure 5. PSOP Structure characterization. (A) SEM microstructure analysis of PSOP; (B) X-ray diffraction pattern.

3.3. Antioxidant Activity of PSOP

Seaweed is increasingly seen as a natural source of antioxidant compounds [64]. Such components can be used in the cosmetics, pharmaceutical, and food industries to protect products from oxidative damage and further deterioration [65]. Three tests were used to assess the reducing and scavenging capacity of PSOP extracted from *Osmundea pinnatifid* algae: DPPH free radical scavenging capacity, iron reduction capacity (RPA), and total antioxidant capacity (TAC). As shown in **Figure 6**, the antioxidant capacity of PSOP is critical and increases with polysaccharide concentration. The data shown in **Figure 6A** revealed that the extract at high concentration (5 mg/mL) exhibited a free radical scavenging activity against DPPH of around 68.9 %. However, BHA shows a high activity of around 97 % at a 0.5 mg/mL concentration. The antioxidant capacity of polysaccharides may be linked to the presence of functional groups such as hydroxyls and carboxyls and to their sulfate content, which scavenges the free radical DPPH [66], [67].

The reducing power test is used to assess the antioxidant efficacy of various substances [68]. As shown in **Figure 6B**, the data reveal that PSOP exhibited a reducing power that correlated with increasing concentrations. A higher absorbance value indicates a more robust reducing capacity. The PSOP polysaccharide showed maximum activity at a concentration of 7 mg/mL (1.33 at OD 700 nm). Nevertheless, this activity was lower than BHA 2.9 at OD 700 nm. The reducing capacity of polysaccharides remains dependent on bioactive elements such as uronic acids, glucuronic acids, and other monosaccharide units [69].

The results of the total antioxidant capacity (TAC) of PSOP in the phosphorus-molybdenum assay are shown in **Figure 6C**. The data reveal that PSOP exhibited different degrees of activity, which

increased with increasing concentration. However, BHA showed higher activity than PSOP at all the concentrations tested. For example, at a concentration of 1 mg/mL, BHA was able to reduce molybdenum Mo (VI) to Mo (V) with a level of 180 $\mu\text{mol/mL}$ α -tocopherol compared to 49 $\mu\text{mol/mL}$ α -tocopherol for PSOP at the same concentration. According to Abdelhedi et al. [70], efficiency (TAC) could be associated with the sulfate levels present in polysaccharides. In addition, Gunasekaran and collaborators have also demonstrated that sulfonic groups in polysaccharides can act as electron donors, reacting to transform free radicals into more stable compounds [71].

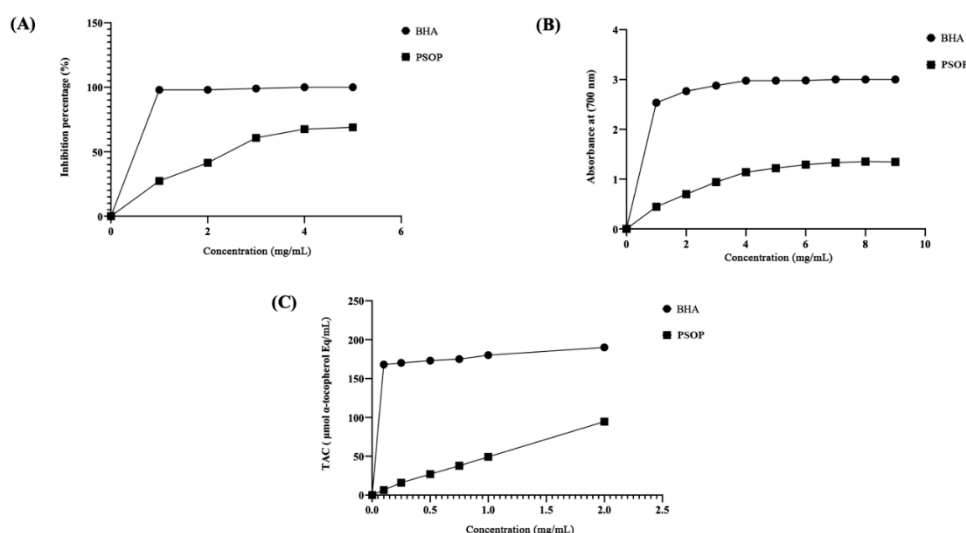


Figure 6. Antioxidant potentials of PSOP. (A) DPPH radical-scavenging activity; (B) reducing power capacity; and (C) total antioxidant capacity.

3.4. Angiogenesis Stimulation Assays

The chorioallantoic membrane (CAM) test is frequently used to examine different molecules' angio-inhibitory and angio-stimulatory potential [72]. Our results have shown that PSOP action is clear and potent. It potentially caused an increase in the number of vessel branches according to the dose. **Figure 7I** and **Table 1** show that CAM treated with different concentrations of PSOP revealed well-developed areas of neovascularization (137.22 % $\pm$ 3.7 %, 200.66 % $\pm$ 2.73 % and 250.66 % $\pm$ 3.49 % vessels for 25, 50 and 100 $\mu\text{g/egg}$, respectively) compared with the group which was not treated (set at 100 %) ($p < 0.05$). Diclofenac (DIC), used as an anti-angiogenic agent to validate the CAM model [73], demonstrated an ability to inhibit the process of new blood vessel formation, thereby reducing neovascularization (71.14% $\pm$ 3.97%) compared to the untreated group ($p < 0.05$). Choriogonadotropin (CG), used as a positive control for its pro-angiogenic role [74], significantly increased blood vessel formation (168.36% $\pm$ 2.72%) compared to the untreated group ($p < 0.05$). Since angiogenesis is a critical factor that promotes new blood vessel formation after chronic or ischemic wounding [75]. Angiogenesis involves phenomena such as hypoxia or inflammation, which lead to the recruitment of inflammatory cells and the release of growth factors that promote the creation of new blood vessels [76]. Among the various factors involved in angiogenesis, tumor necrosis factor- α (TNF- α) stands out as a pro-inflammatory cytokine. This cytokine acts on endothelial cells, producing vascular endothelial growth factor (VEGF) [77]. VEGF promotes cell proliferation, activates the expression of specific genes, stimulates nitric oxide production, and supports cell survival [78], [79]. Numerous studies have shown that polysaccharides can regulate the expression and activity of growth factors involved in angiogenesis, including VEGF [80], [81]. Therefore, our results indicate that PSOP may influence angiogenesis by activating the inflammatory process and increasing levels of VEGF and TNF- α .

Table 1. Percentage of blood vessels in CAM assay.

Groups	Control	Diclofenac (50 µg/egg)	Choriogonadotropin (30 µg/egg)	PSOP (25 µg/egg)	PSOP (50 µg/egg)	PSOP (100 µg/egg)
Vessel Number (%)	100 ^a	71.14 ± 3.97 ^b	168.36 ± 2.72 ^c	137.22 ± 3.7 ^d	200.66 ± 2.73 ^e	250.66 ± 3.49 ^f

Data are presented as percentages, and the control group is set to 100 %. Values are expressed as means ± SD for 6 eggs in each group. ^{a, b, c, d, e, f} Indicate significant differences between different groups (p < 0.05).

3.5. Quantitative Analysis of Vascular Network

Figure 7II shows the version processed by AngioTool software. Vessel outlines are marked in yellow; vessels are marked in red, and junctions are marked in blue. The results indicate that the density of blood vessels in the PSOP-treated group is more significant than that in the control group, which reveals the pro-angiogenic nature of PSOP.

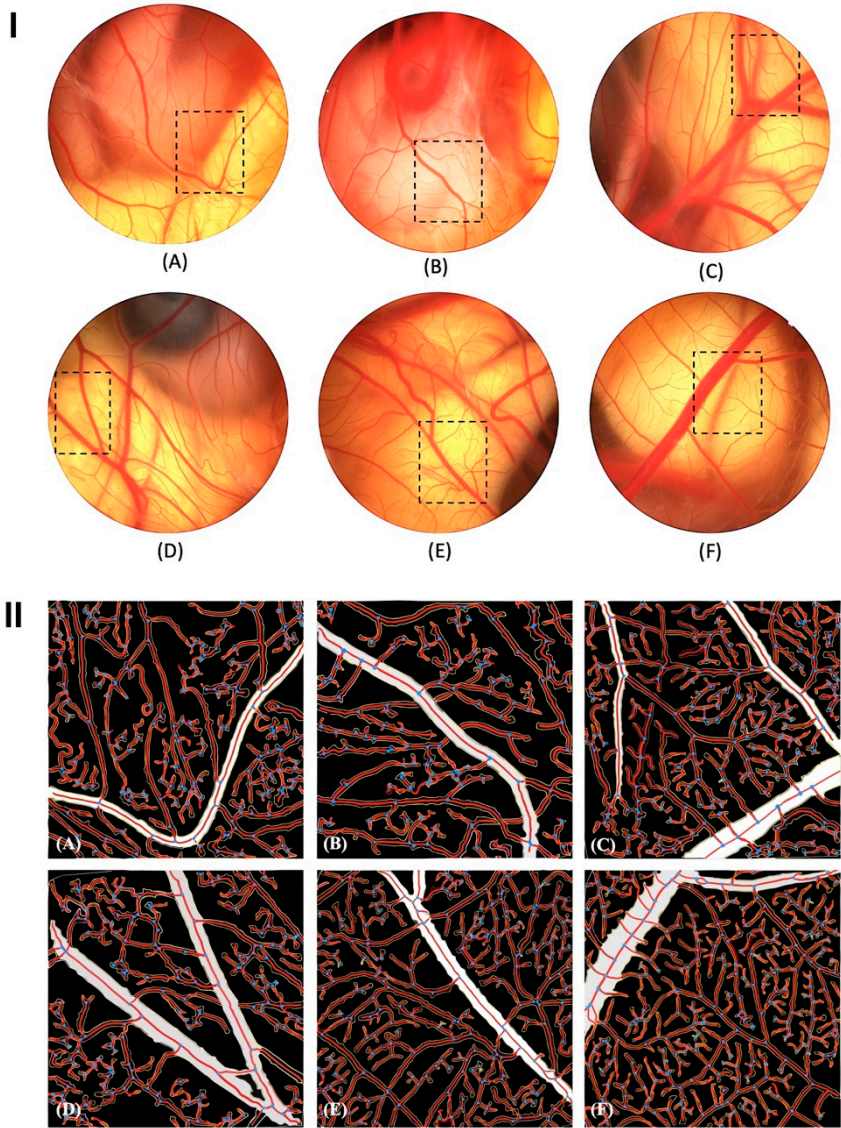


Figure 7. PSOP stimulated neovascularization of the chorioallantoic membrane in 12-day-old fertilized eggs. (A) distilled water (control); (B) Diclofenac (50 µg/egg); (C) Choriogonadotropin (30

µg/egg); (D) PSOP (25 µg/egg); (E) PSOP (50 µg/egg) and (F) PSOP (100 µg/egg). (I): Original CAM images; (II): Resulting images after processing with AngioTool v 0.6a. The ring fields were photographed (magnification, ×10).

Quantitative analysis of blood vessels in **Figure 8** shows that the concentrations of 50 and 100 µg/egg of PSOP extract and choriogonadotropin (CG) result in a significant increase in vascular surface area, vessel length, and diameter, as well as the number of junctions, compared to the control and the negative control diclofenac (DIC). Moreover, the analysis showed no significant difference between treatment (25 µg/egg) with PSOP extract and control. As expected, a lower lacunarity value has been identified in the PSOP-treated groups (50 and 100 µg/egg) and the positive control compared to the control and the negative control groups. The high lacunarity values signaled that empty spaces in the vascular network architecture in these groups were more frequent. According to the previous data, we deduced that PSOP extract produced remarkable results in forming new vessels. Better vascularization improves oxygen and nutrient delivery to the wound site, essential for tissue regeneration and rapid healing.

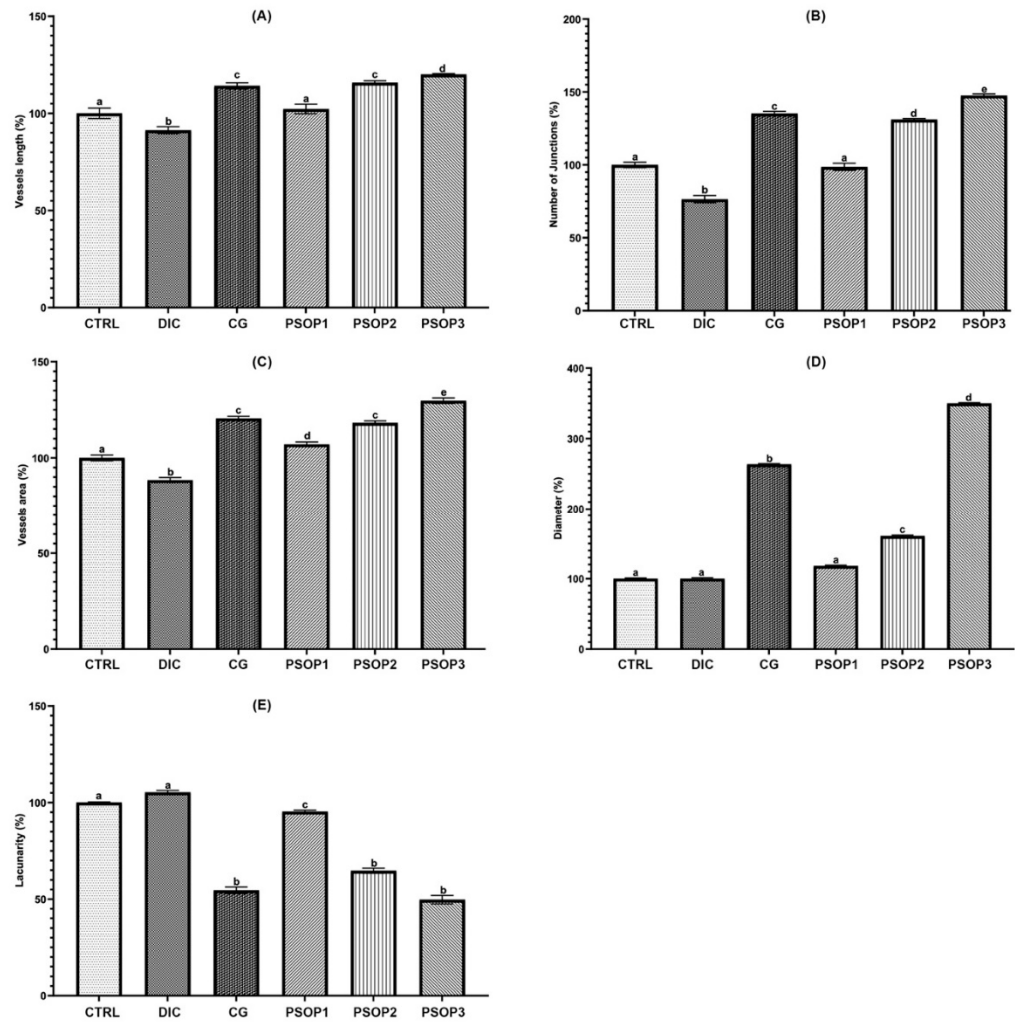


Figure 8. Effect of treatments on the density and morphology of the vascular network in the chorioallantoic membrane (CAM). (A) Vessel area, (B) Vessel length, (C) Vessel diameter, (D) Number of junctions, and (E) Lacunarity. Data are presented as percentages, and the control group is set to 100 %. CTRL: Control; DIC: Diclofenac; CG: Choriogonadotropin; PSOP1: 25 µg/egg; PSOP2: 50 µg/egg; PSOP3: 100 µg/egg. The data represents the mean ± SEM of six samples in each group. ^{a,b,c,d} indicate significant differences between different groups (p < 0.05).

3.6. In Vivo Experimental Study

3.6.1. Morphological Evaluation

The wound contraction level was visualized and evidenced by capturing images at different time intervals (1, 5, 7, and 11 days). As shown in **Figure 9**, day 1 illustrates the initial state of the wounds immediately after induction and application of the various treatments. All wounds were homogeneous in appearance, with dark red coloration, well-defined margins, and visibly damaged tissue, demonstrating wound uniformity in all groups. No significant differences between the groups are discernible at this stage, which is to be expected. These observations serve as a benchmark for assessing treatment efficacy on subsequent days. On day 5 of treatment, wounds in the PSOP and Cytol Centella groups showed brown discoloration, indicating the formation of a crust resulting from the onset of blood coagulation healing. Both treatments showed a marked improvement in healing compared to the untreated groups (saline and glycerol), with a significant reduction in wound size and a less inflamed appearance. On day 7, wounds treated with saline and glycerol showed less advanced healing progression than those treated with PSOP and Cytol Centella, which delayed tissue regeneration. On the 11th day, no scarring was observed in the group treated with PSOP.

Nevertheless, an open wound and scab fragments persisted in the group treated with saline and glycerol. The morphological assessment indicates that treatment with PSOP accelerates the healing process, restoring the skin's initial structural color. Indeed, reactive oxygen species (ROS) are often linked to delayed wound healing. When ROS are overproduced, without an effective antioxidant response, cell membranes, proteins, and DNA suffer oxidative damage, leading to prolonged inflammation and extracellular matrix degradation, which delays wound healing [82]. It is well known that polysaccharides can neutralize ROS. Their action involves reducing oxidative stress by scavenging oxidizing free radicals, thus preventing oxidative damage to cells and tissues [83]. This suggests that the antioxidant properties of PSOP help to protect cells against oxidative stress, thereby reducing wound stress and promoting faster, more effective wound healing.

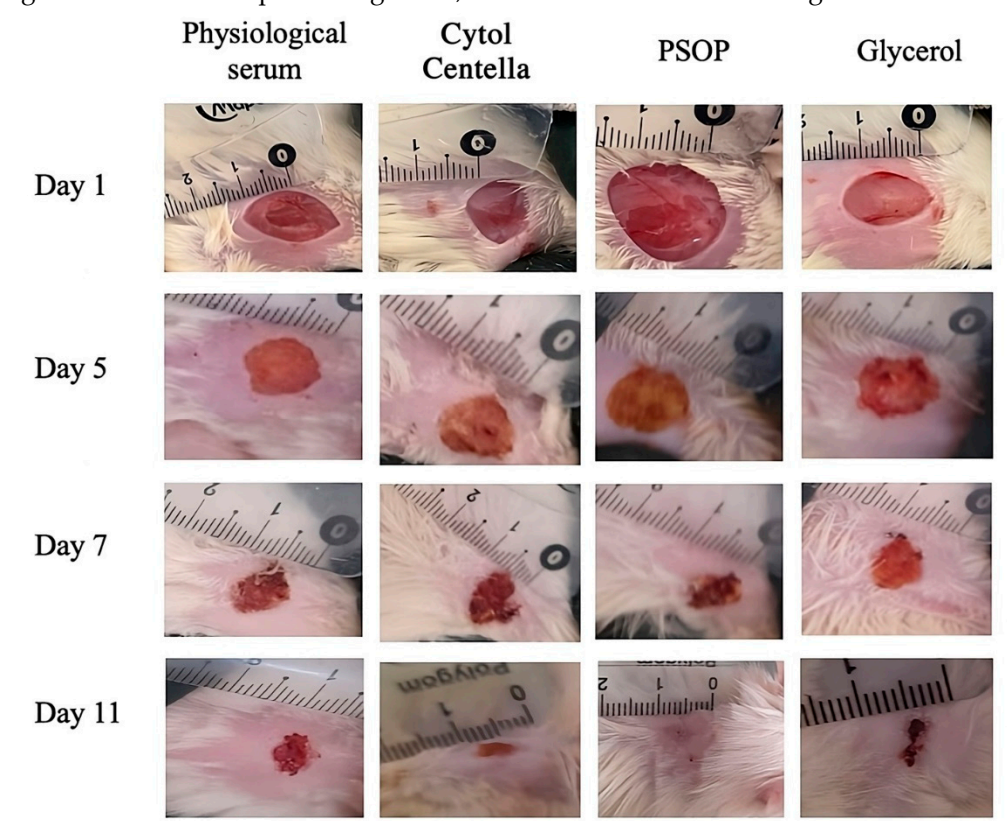


Figure 9. Representative photographs of the macroscopic appearance of 1.5 cm² wounds excised on rats at days 1, 5, 7, and 11 of the group treated with physiological serum, glycerol, Cytol Centella, and PSOP hydrogel.

3.6.2. Assessment of Wound Area

Epithelialization is an essential phase in wound healing, during which epithelial cells migrate upwards to cover and repair the wounded area [84]. In this way, the healing potential of the treatments was assessed throughout the 11-day experimental period by punctually measuring the size of the injured areas. **Figure 10** shows that the groups treated with PSOP and Cytol Centella showed significantly greater healing capacity than the untreated groups (glycerol and saline). The PSOP-treated group achieved complete wound closure (100%) by day 11, indicating optimal efficiency for tissue regeneration. The Cytol Centella group also showed notable performance, with a wound reduction of $90.67\% \pm 0.27\%$, albeit lower than with PSOP.

In comparison, the glycerol and saline showed slower healing, with respective closure rates of $70.49\% \pm 1.09\%$ and $66.59\% \pm 1.10\%$. These results suggest that using PSOP enables faster and more complete restoration of skin structure, illustrating its potential as a pro-healing agent. The epithelialization process is strongly influenced by wound conditions, particularly the moisture content of the wound environment. A moist environment encourages epidermal cell migration, accelerates re-epithelialization, and maintains high levels of proteinases and growth factors, all of which are essential for ideal wound healing [85]. In this sense, polysaccharides derived from algae play an important role in maintaining a moist wound-healing environment. Thanks to their hydrophilic properties, biocompatibility, and ability to retain moisture [86].

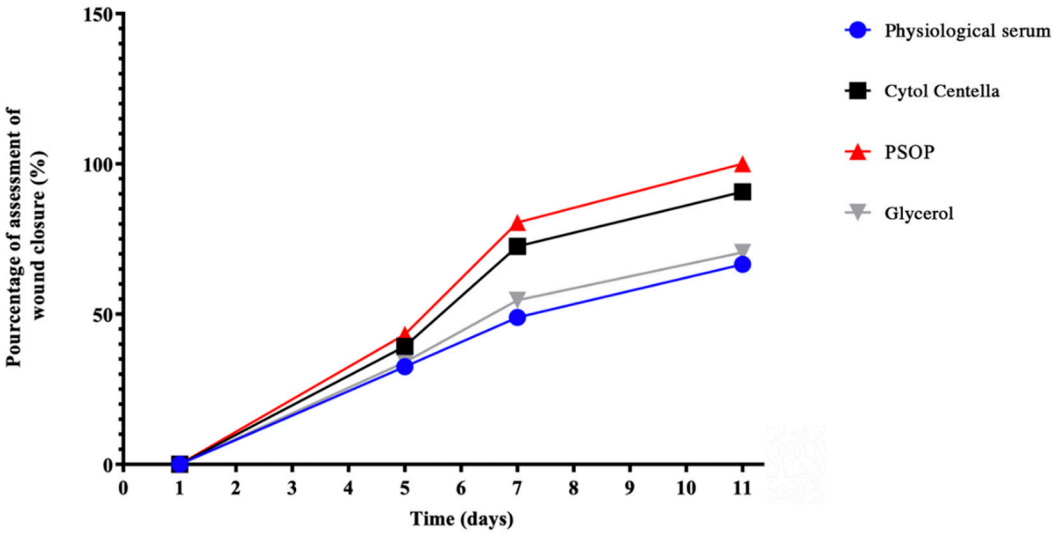


Figure 10. Percentage of wound area contraction of different groups of rats. Each value represents the mean $\pm$ SEM of results from 6 rats. ^{a,b,c, d} In the same column indicate significant differences ($p < 0.05$).

	5 days	7 days	11 days
Physiological serum	32.5 $\pm$ 1.24 ^a	48.9 $\pm$ 2.02 ^a	66.59 $\pm$ 1.59 ^a
Cytol Centella	39.3 $\pm$ 1.41 ^b	72.54 $\pm$ 2.85 ^b	90.67 $\pm$ 2.08 ^b
PSOP	43.22 $\pm$ 1.92 ^c	80.45 $\pm$ 3.01 ^c	100 ^c
Glycerol	34 $\pm$ 1.74 ^d	54.62 $\pm$ 2.07 ^d	70.49 $\pm$ 1.27 ^d

3.6.3. Hydroxyproline and Collagen Regeneration

To confirm the regeneration of skin tissues, wound area biopsies were evaluated to estimate tissue hydroxyproline levels in all groups. As illustrated in **Figure 11**, the PSOP-treated group showed high hydroxyproline levels (750.81 ± 21 mg/g tissue) compared with the other groups (162.53 ± 7.71 , 202.45 ± 14.8 and 626.5 ± 18.95 mg/g tissue for saline, glycerol and Cytol Centella, respectively) ($p < 0.05$). In reality, high levels of hydroxyproline indicate the synthesis of collagen fibers and induce the proliferation and remodeling of the wound [87]. According to Zhang et al. [88], they have shown that polysaccharides can promote the expression of interleukin-1 beta ($IL-1\beta$), which in turn stimulates VEGF production. This cascade of signals promotes angiogenesis and collagen synthesis, which is crucial for tissue regeneration and wound closure. Another factor influencing hydroxyproline production is the PI3K/Akt/mTOR signaling pathway. This pathway plays a key role in regulating cell proliferation and survival, as well as collagen synthesis [89]. Some studies have shown that polysaccharides can activate these signaling pathways, which could indirectly influence hydroxyproline production and, consequently, collagen stability and formation [90], [91]. Our experimental data and available literature evidence support the hypothesis that PSOP could modulate these pathways, thereby contributing to collagen synthesis and skin tissue healing.

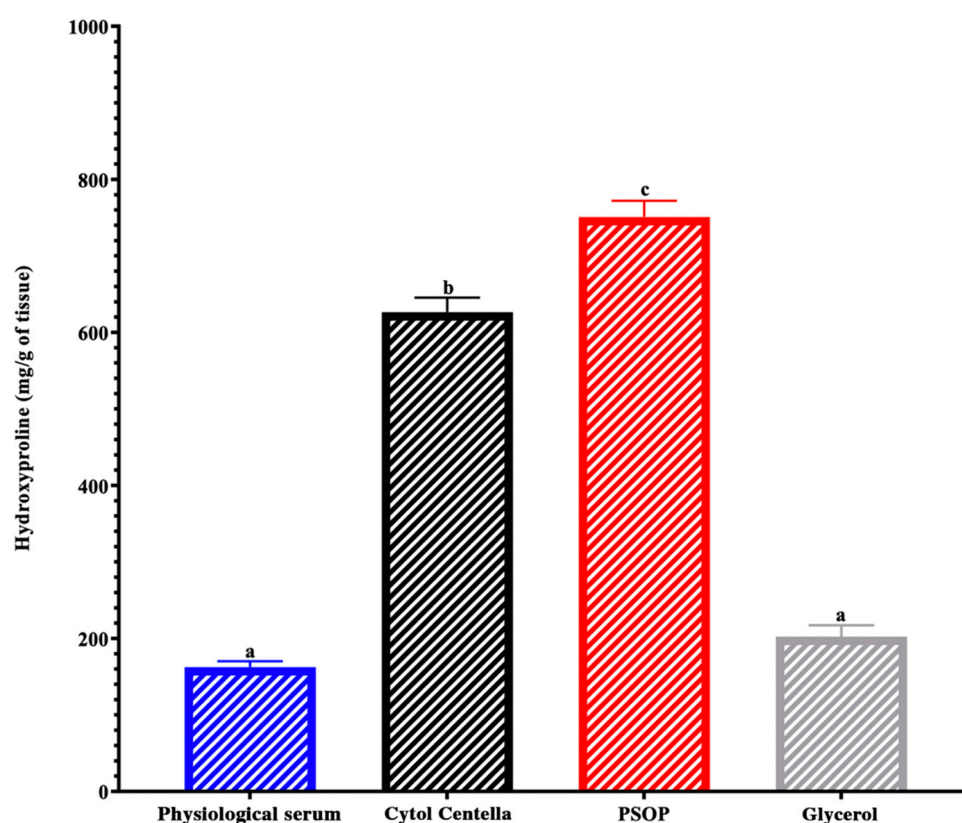


Figure 11. Hydroxyproline concentrations in biopsies from experimental rat wounds. Different letters on each bar indicate significant differences between treatments ($p < 0.05$).

3.6.4. Histological Evaluation

Re-epithelialization corresponds to the restoration of the epithelium after a wound, thanks to the proliferation and migration of epithelial cells on the wound surface, creating a protective cover over regenerating tissue. In addition, granulation tissue formation, neovascularization, and collagen deposition are fundamental mechanisms identified throughout the cutaneous wound healing process [92]. To assess the level of wound healing, skin tissue from treated and untreated groups was stained with hematoxylin-eosin and examined at the end of the experiment. The evaluation focused on re-epithelialization, collagen and fibroblast formation, neovascularization, and the deposition of

inflammatory cells. As illustrated in **Figure 12**, microscopic photographs of the untreated groups (saline and glycerol) reveal the persistence of an invasive inflammatory infiltrate with necrosis of the epidermis and dermis, and we also observed low collagen synthesis, a consequence of incomplete repair and re-epithelialization. Nevertheless, the wound tissue sections of the PSOP and Cytol Centella treated groups demonstrated denser epithelization, neo-vascularization, as well as the disappearance of inflammatory cells, which confirms our earlier results regarding the determination of hydroxyproline in wound biopsies.

Histological sections from the PSOP-treated group showed complete healing, supported by accelerated cell proliferation, protection against oxidative damage, and reduced inflammation. The results also confirmed the PSOP's pro-angiogenic potential, which seems capable of stimulating the expression of VEGF, thereby promoting the proliferation of endothelial cells and fibroblasts.

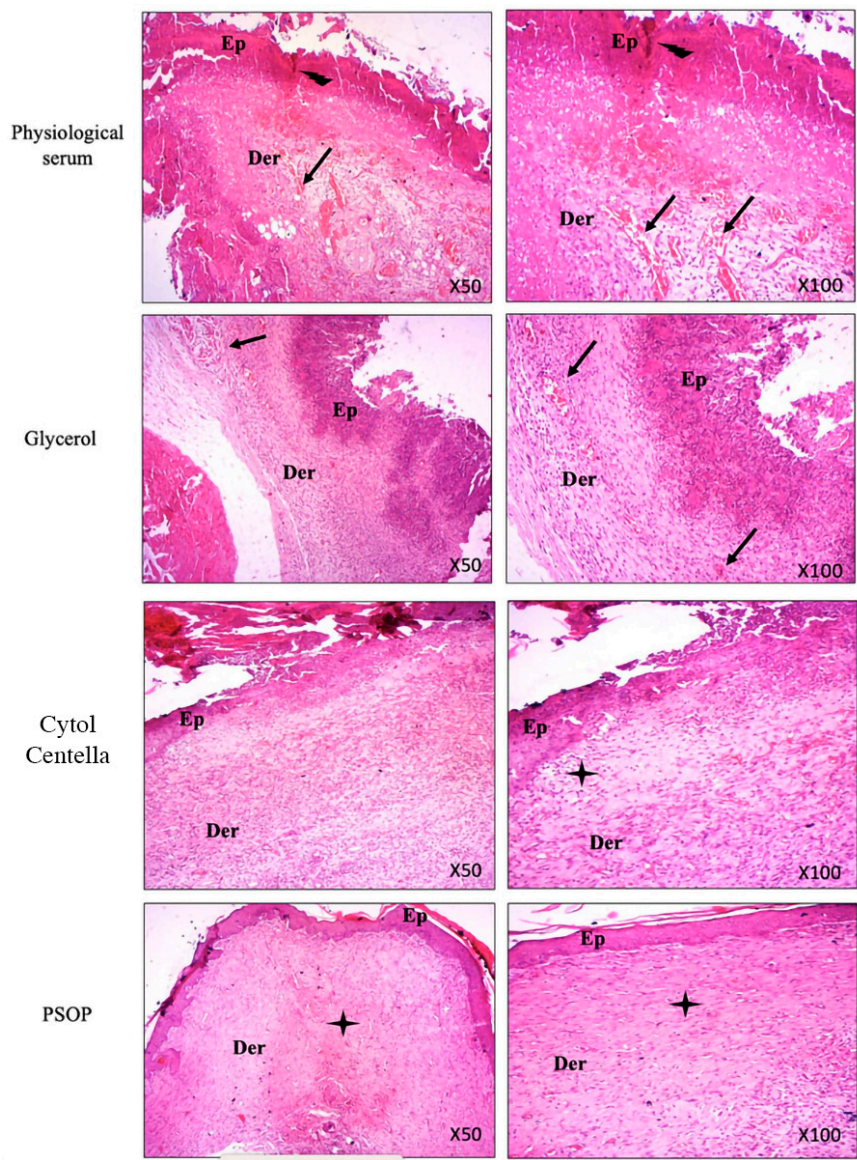


Figure 12. Representative microphotographs of epidermal and dermal wound structure on day 11 in rats treated with saline, glycerol, Cytol Centella, and PSOP hydrogel. Tissues were visualized at 50 and 100× magnification. Arrows indicate the following: E: epidermis and D: dermis. ★ : Ulceration
: Inflammatory infiltrate : Fibers of collagen

3.7. Computational Findings

The PSOP-identified monosaccharides showed different affinities to two targeted receptors (Table 2). Nevertheless, they all had negative binding affinities, which might support their biological effects. The predicted binding affinities ranged between -5.6 and -6.4 kcal/mol for COX-2 and -4.1 and -4.6 kcal/mol for VEGF. Such variations have been reported to be the result of 3D chemical structures of the ligands ([34], [33], [93]). The identified monosaccharides established acceptable molecular interactions with both targeted receptors, including four to six conventional H-bonds (Table 3). The established molecular interactions concerned several key residues. For instance, fructose while complexed with COX-2, fructose interacted twice with ASN³⁹ and once with each of CYS⁴⁷, GLU⁴⁶⁵, CYS⁴¹, and GLY⁴⁵ (Table 3 and Figure 13). While complexed with VEGF, glucose interacted twice with LEU³⁹ and once with THR³⁶, LEU³⁵, and LYS⁴⁵. PSOP units were also deeply embedded in the pocket region of the targeted receptors and showed a vicinity of up to 2.072 Å only.

Deep embedding (<2.5 Å), as those of the current study, was previously reported to enhance the bioactivities such as antiproliferative, anti-inflammatory, toxicity alleviation, and antimicrobial effects ([32], [93], [34], [35]). The binding affinities, the deep embedding, and the revealed molecular interactions of PSOP building blocks indicate that the pro-angiogenic and wound-healing potential effects of PSOP chemicals are thermodynamically possible. These predicted bioactivities had already been confirmed by the *in vitro* study in rats. Our results support the beneficial effects of natural-derived compounds, phytotherapy, and medicinal plants, including algae ([94], [93], [35]).

Table 2. Binding affinity of root mean square deviation of the *O. pinnatifida* identified monosaccharides with the two targeted receptors: 1cx2 and 2c7w for cyclooxygenase 2 (COX-2) and vascular endothelial growth factor (VEGF), respectively.

Compound No.	COX-2	VEGF
	(1cx2)	(2c7w)
Binding Affinity (kcal × mol ⁻¹)		
Arabinose	-5.7	-4.5
Fructose	-6.4	-4.5
Glucose	-6.3	-4.6
Glucuronic acid	-5.6	-4.4
Xylose	-6.1	-4.1
RMSD (lower – upper)		
Arabinose	0.0 - 30.13	0.0 - 31.90
Fructose	0.0 - 30.82	0.0 - 9.08
Glucose	0.0 - 22.70	0.0 - 9.14
Glucuronic acid	0.0 - 41.78	0.0 - 14.83
Xylose	0.0 - 9.86	0.0 - 30.91

Table 3. Number of conventional H-bonds, closest interacting residues and distance to closest interacting residue (Å) of the *O. pinnatifida* identified monosaccharides with the two targeted receptors: 1cx2 and 2c7w, for cyclooxygenase 2 (COX-2) and vascular endothelial growth factor (VEGF), respectively.

Saccharide	No.	Closest		
	Conventional H-Bonds	Interacting Residues	Closest residue (Distance, Å)	No. closest interacting residues
Cyclooxygenase 2 (COX-2)				
Fructose	6	CYS ⁴⁷ , ASN ³⁹ , GLU ⁴⁶⁵ , ASN ³⁹ , CYS ⁴¹ , GLY ⁴⁵	GLU ⁴⁶⁵ :OE1 (2.072)	5
Vascular Endothelial growth factor (VEGF)				
Glucose	4	LEU ³⁹ , THR ³⁶ , LEU ³⁵ , LYS ⁴⁵ , LEU ³⁹	LEU ³⁹ :HN (2.247)	4
Bold residues: amino acids interacting with conventional H-bond				

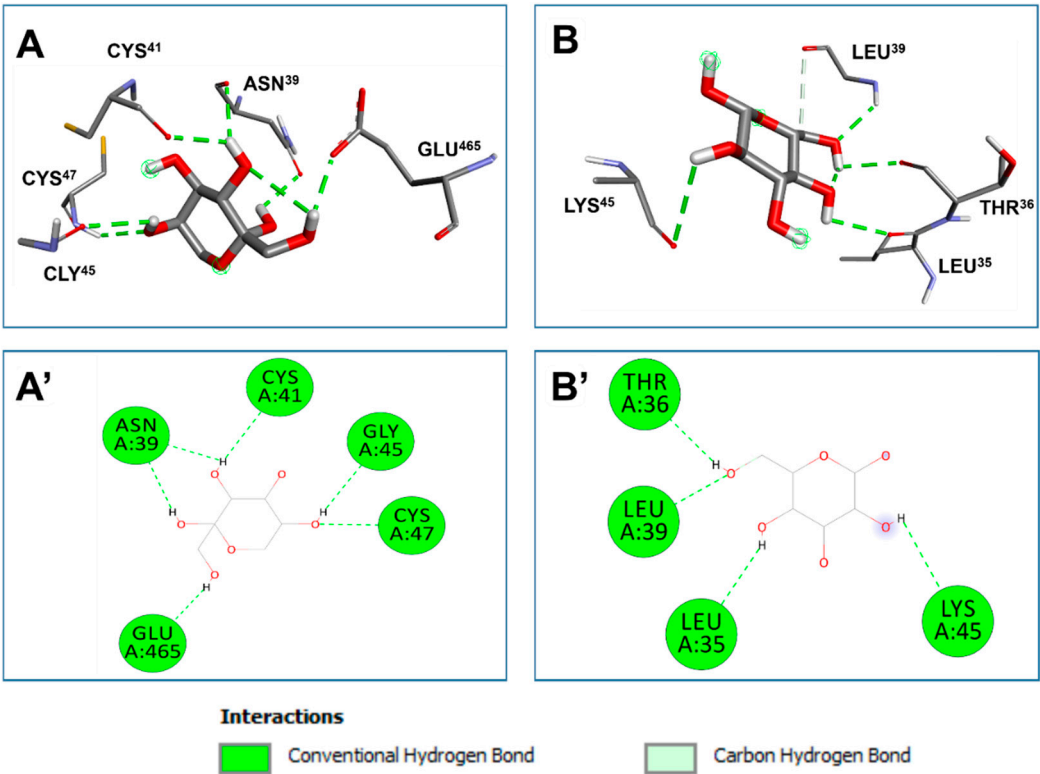


Figure 13. 3D illustration of the pocket regions (A and B) and resulted diagrams of interactions (A' and B') of the *O. pinnatifida* identified monosaccharides with the two targeted receptors: 1cx-2 and 2c7w for cyclooxygenase 2 (COX-2, A) and vascular endothelial growth factor (VEGF, B), respectively.

4. Conclusions

This is an unprecedented report on the antioxidant activity, healing, and pro-angiogenic properties of PSOP extracted from the red alga *Osmundea pinnatifida*. The PSOP is genuinely and potentially interesting due to the antioxidant activities confirmed by various tests (DPPH, TAC and RPA). These activities include eliminating the DPPH free radical, reducing iron (III), and converting molybdenum Mo (VI) to Mo (V). The findings of our study show that PSOP has a pro-angiogenic effect when tested using the CAM assay. This effect was further confirmed by observing an accelerated wound healing process in Wistar rats after 11 days, involving angiogenesis, fibrotic tissue formation, remodeling, and reduced healing time using the circular excision model. Our combined experimental and computational results suggest that PSOP could be exploited as a natural bioactive material to improve the healing of chronic wounds, such as diabetic ulcers, burns, or even to promote vascularization during skin grafting procedures, etc.

Author Contributions: Conceptualization: AH, IY and IBA; Methodology: ZB, ME, AF, HBS and SE; Software: ZB, MK and RB; Validation: AH, IBA and SH; Supply of reagents and materials: IBA, SH and SE; Writing – original draft preparation, ZB; Writing – review & editing: JMP, HK and RB; Visualization: IBA, RB, JMP and HK. All authors have read and accepted the published version of the manuscript.

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Ethical Statements: All animal protocols complied with European Union legislation (Directive 2010/63/EU) on protecting animals used for scientific purposes and were approved by the local ethics committee.

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