

Full Length Research Paper

***In vitro* and *in vivo* investigations of the wound healing effect of crude *Spirulina* extract and C-phycoerythrin**

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The aim of this study was to evaluate the influence of crude *Spirulina* extract and C-phycoerythrin (C-PC) isolated from crude *Spirulina* extract on cultured human keratinocyte, using *in vitro* and *in vivo* models of wound healing. *Spirulina* has been used as a nutraceutical and source of potential pharmaceuticals, however it is not known which component of the cyanobacteria is effective for wound healing. In *in vitro* model, cultured human keratinocyte were used to investigate the effects of crude *Spirulina* extract (PSE) and C-phycoerythrin (C-PC) extracts on processes involved in keratinocyte proliferation, regeneration and migration. Keratinocyte proliferation and regeneration were monitored by the colorimetric (MTT) assay and migration was monitored in relation to the closure of a denuded area scratched in a confluent monolayer. On the other hand, in *in vivo* model using Sprague-Dawley male rats, effects of PSE and C-PC on tissue regeneration were investigated. Results of *in vivo* wound healing study were monitored by means of histological examinations. PSE extract showed the best growth stimulation at 33.5 µg/mL dose of treatment, which revealed a cell viability ranging from 100 to 270% after 72 h. Cell viability was also good for C-PC and was measured as high as 213%. Cell viability and proliferation difference between PSE and C-PC were observed not to be significant ($p > 0.05$) at the range of doses (33.5 to 0.0335 µg/mL) studied. In *in vivo* efficiency of the PSE and C-PC, it was observed that 1.25% C-PC has a better effect on the 7th day compared to other preparations.

Key words: *Spirulina* extract, c-phycoerythrin, *in vitro*, *in vivo*, Sprague-Dawley rats, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), tissue regeneration.

INTRODUCTION

Wound healing is a complex but highly regulated and dynamic interaction between orderly expressed cell types, extracellular matrix and an array of soluble mediators. The process of wound repair involves keratinocyte and fibroblast proliferation, regeneration and neovascularization. In the cutaneous setting, wound healing is traditionally divided into three stages: inflammation,

cellular proliferation, tissue remodeling and maturation (Devalaraja et al., 2000).

For treatment of burns alone, five billion US dollars are spent every year in the world. Over the last two decades, substantial advances have been made in understanding the molecular and cellular biology as well as the biochemistry, physiology and pathology of wound healing. Although a number of advanced techniques such as treatment with recombinant growth factors, gene therapy, use of skin equivalents, and grafting of cultured keratinocyte have been developed, the application of these techniques is often not feasible in developing

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countries because of limited resources and the need for specialized laboratories. Burns and trauma wounds are common and at the same time they constitute a major problem in developing countries because the rate of severe complications is high and financial resources are limited. In these countries, one important aspect in treatment of burns and wounds is the use of local herbal remedies, not only by rural communities, but also by the authorities in charge of health. New formulations of old remedies are developed and distributed. However, there is a need for controlled clinical and scientific investigations of some of the most promising ones (Phan et al., 2001).

Different medicinal plants and their medicinal values are widely used for various ailments throughout the world. Current traditional remedies for cure in various ailments including skin treatments have been on the increase (Sharma et al., 2009; Mahmood et al., 2010; Coopoosamy, 2010; Odimegwu et al., 2008).

As part of our ongoing search for wound healing from medicinal sources, we analyzed *Spirulina* (Arthospira). The cyanobacteria *Spirulina* which is consumed in daily diets of natives in Africa and America has been found to be a rich natural source of proteins, carotenoids and other micronutrients (Farooq et al., 2004) Interest in *Spirulina* increases due to the fact that it is a nutraceutical and that it is also a source of potential pharmaceuticals. As a result of this fact, it has many biological activities. Recent studies have demonstrated antioxidant, anti mutagenic, antiviral, anticancer, anti-allergic, immune enhancing, hepato-protective, blood vessel relaxing and blood lipid-lowering effects of *Spirulina* extracts (Kim et al., 1998; Subhashini et al., 2004).

The biological and pharmacological properties of *Spirulina* are attributed mainly to calcium-spirulan (Ca-SP) and C-phycoerythrin (C-PC) (Subhashini et al., 2004). Ca-SP, a sulfated polysaccharide consists of rhamnose, ribose, mannose, fructose, galactose, xylose, glucose, glucuronic acid, galacturonic acid, sulfate and calcium. It inhibits *in vitro* replication of enveloped viruses such as herpes simplex virus type 1 (HSV-1), human cytomegalovirus, measles virus, mumps virus, influenza A virus and human immunodeficiency virus type 1 (HIV-1) (Hernandez-Corona et al., 2002; Rahman et al., 2006). It has been shown that Ca-SP activates heparin cofactor II which is a physiological inhibitor of thrombin (Hernandez-Corona et al., 2002; Rahman et al., 2006; Kaji et al., 2002; Qishen et al., 1988). Ca-SP exhibits *in vitro* anti-thrombin activity by a unique mechanism which is different from that of heparin (Hernandez-Corona et al., 2002; Rahman et al., 2006; Kaji et al., 2002; Qishen et al., 1988). It can well be an origin of anti-thrombogenic, fibrinolytic and anti-atherogenic medicines (Hernandez-Corona et al., 2002; Rahman et al., 2006; Kaji et al., 2002; Qishen et al., 1988). The presence of polysaccharide in *Spirulina* not only increases the initial rates of damaged DNA excision and the DNA synthesis, but also

postpones the saturation of two important reactions: excision and repair of DNA synthesis (Qishen et al., 1988).

C-PC is one of the major light harvesting biliproteins of *Spirulina* and is of great importance because of its various biological and pharmacological properties. This pigment is known to be hepatoprotective, antioxidant, radical scavenger, anti-arthritis, anti-inflammatory, anti-tumor and immunity boosting activities and is used not only as nutrient ingredient and natural dyes in food and cosmetics industries but also as fluorescent markers in biomedical research (Reddy et al., 2003; Li et al., 2005; Patel et al., 2005; Hirahashi et al., 2002). It is also a potent profibrinolytic protein in the vascular endothelial system (Madhyastha et al., 2006). It protects the renal cell against oxalate-induced injury and may be a nephroprotective agent (Farooq et al., 2004).

The aim of this study is to investigate the effect of the crude *Spirulina* extract (PSE) and C-phycoerythrin (C-PC) on the human keratinocyte proliferation and on their migration in a "wounded" cultured monolayer (*in vitro* model) and then to examine their wound healing effect regarding tissue regeneration in *in vivo* animal model using Sprague-Dawley male rats.

MATERIAL AND METHODS

Production of *Spirulina platensis*

The origin of the strain of *S. platensis* used in this study goes back to Peru Parachas. The pure culture was obtained from Ege University microalgae culture collection, Ref. No: EGE-MACC-31. The cyanobacteria were produced in raceway ponds agitated by a paddle wheel under sunlight in Zarrouk's medium and harvested by filtration method. The bioreactor used for growing the inoculum culture of pond production was illuminated by 8 florescent lamps, two on the top, two at the bottom and four at both sides of the bioreactor. When the culture reached 140 L in volume, it was transferred to the open raceway ponds located in the plant. The cyanobacteria were harvested by filtration lay out on the drying trays and were dried in an aerated dryer (Vonshak, 1997).

Crude extract preparation

The crude extract of the cells was prepared by freezing-thawing method. Filtered cells produced in the raceway ponds were frozen and thawed in order to disrupt their membrane, and afterwards it was extracted by hot water. The extract frozen at 24°C was thawed quickly in phosphate buffer saline (PBS, pH 7) at 4°C. Three cycles of freezing-thawing were performed and the process was followed by centrifugation (9,000 rpm, 30 min, Hettich Rotina 35 R Germany) in order to precipitate the solid particles. The aliquot was sterilized by filtration gradually (0.8, 0.45 and 0.22 µm, Sartorius Germany). The supernatant was aliquoted into 1.5 mL of sterile bottles, lyophilized (Christ 1.8 B-Plus, Germany) and kept at -20°C until used (Minkova et al., 2003).

Extraction and purification of C-PC

Extraction and purification processes were carried out according to the method laid out by Zhang and Chen (1999). 10 g of lyophilized

S. platensis powder was suspended in 1000 mL of 1.5% $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (w/v) aqueous solution. The suspension was agitated over night at 4°C and centrifuged at 9,000 rpm for 45 min (Hettich Rotina 35 R Germany). The precipitate was removed and then left for saturation with 40% ammonium sulphate overnight at 4°C. The precipitate was removed by centrifugation for 20 min at 13,000 rpm. The same process was repeated with 70% ammonium sulphate saturation step. The final precipitate was dissolved in 20 mL of 0.05 M PBS (pH 7) and dialyzed against the same buffer solution overnight. C-PC containing dialyzed samples was chromatographed thorough DEAE–sepharose CL-6B (Amersham Biosciences, Switzerland) columns (2.5 × 15 cm).

The C-PC containing samples were eluted with the ion concentration gradient (0 to 0.25 M NaCl, 1 mL/min). The salt was removed by washing with pH 7, 0.002 M sodium phosphate buffer solution (0.5 mL/min) in the Sephadex-G 100 (Sigma-Aldrich, Germany). A_{620}/A_{280} ratios were considered for choosing the fractions; the ratios above 4 were collected in 1 mL of tubes. The UV visible spectrums of the C-PC samples were measured with the Varian Cary 300 Bio UV/Vis spectrophotometer (Australia). 620 nm is the maximum wavelength that C-PC can absorb (10). A_{620}/A_{280} ratios were evaluated for the purity of the fractions; where by resulting values of 4 and higher were considered as pure and were collected. After lyophilization, the samples were kept at -20°C (Vonshak, 1997).

Dry weight determination

The “ $P1 = P3 - P2$ ” equation was used to calculate the dry weight of crude *S. platensis* extract in these experiments, where P1 refers to dry weight (mg), P2 refers to the weight of the bottles and P3 is the weight of the bottle with lyophilized extract.

Cell viability and proliferation assays

Human keratinocytes (coded HS2) obtained from Animal Cell Culture Collection (HUKUK, Sap Institute, Ankara, Turkey) were produced with DMEM-HAM's/F-12 (Biochrom, Germany) medium supplemented with 10% fetal calf serum (FCS, Biochrom, Germany) and were incubated at 37°C with 5% CO_2 in humidified atmosphere. Cells in exponential growth phase were placed in 96-well plates so as to make 6,000 cells/wells. After 24 h of incubation and adding sample solutions in concentrations ranging from 0.0335 to 33.5 $\mu\text{g}/\text{mL}$ in each well, respectively, they were incubated for 72 h. Groups were treated with DMEM-HAM's/F-12 as negative control and DMEM-HAM's/F-12 with EGF (Sigma, USA) (10 ng/mL) as positive control.

Cell proliferation was determined by adding 0.5 $\mu\text{g}/\text{mL}$ per well, prepared as a sterile stock-solution of 5 mg/mL in Dulbecco's-phosphate buffered saline (DPBS, Gibco, USA), diluted 1:10 with medium prior to use. Medium was removed 4 h later and blue formazan crystals dissolved in 200 μl 100% dimethylsulfoxide (DMSO, Sigma, USA) per well. Quantities of blue formazan product were measured at 570 to 690 nm using UV visible microplate reader spectrophotometer (Molecular Devices, Versamax, Tunable Microplate Reader, USA). For human keratinocytes, strong correlations between numbers of cells present and amounts of MTT formazan product were observed. The data were obtained from three independent assays using three wells for each assay (Sevimli-Gur et al., 2011).

In vitro wound healing, proliferation and migration method

Cells were placed in 24-well plates so as to make 2×10^5 cells/well and were cultured until covering the surface completely. In a circu-

lar zone of 5 mm diameter cell layer making use of a specially designed tool for artificial *in vitro* wound formation by a sterile teflon bar that removes cells from the culture surface, a wound was formed by scratching carefully (Arikan et al., 2007). After the formation of the wound, cell debris was removed by discarding the medium and washing the wells 4 times with DPBS. Medium containing tenfold concentration of sample solutions ranging from 0.0335 to 33.5 $\mu\text{g}/\text{mL}$ was added to the cultures in which the wound models were formed. As negative control groups DMEM-HAM's/F-12 medium with 10% FCS, DMEM-HAM's/F-12 medium without FCS and Hank's balanced salt solution (HBSS, Biochrom, Germany) were used. For the positive control group DMEM-HAM's/F-12 supplemented with 10 ng/mL of epidermal growth factor (EGF, Sigma, USA) was used. At the end of 72 h of incubation at 37°C with 5% CO_2 , cells were fixed with 4% paraformaldehyde and stained with Giemsa and/or by hematoxyline-eosine (HE). Healing in the wound zone was photographed. After transferring the pictures to a computer, the number of cells that were formed as a result of migration and proliferation were determined (Sevimli-Gur et al., 2011).

Animal model and surgical procedure

The experimental protocol was approved by the Institutional Committee on the Care and Use of Laboratory Animals, Hacettepe University, Ankara, Turkey. The study was permitted by the Institutional Animal Ethics Committee and was performed according to the international rules considering the animal experiments and biodiversity right. A total of 21 Sprague-Dawley male rats (outbreed stock from own breeding colony) aged 12 weeks were used in this study. The rats weighed 250 to 300 g at the beginning of the experiment. They were socially housed in Eurostandard type IV polycarbon cages ($w \times h \times d = 380 \times 200 \times 590$ mm) with standard rat food pellets (Korkutelim Limited, Turkey) and water available *ad libitum*. The colony room was maintained at a temperature of $21 \pm 2^\circ\text{C}$, a relative humidity range of 40 to 50% and on a 12 h light/12 h dark cycle.

The rats were randomly assigned to three groups, each consisting of 7 according to time points. All surgical procedures were performed under general anesthesia by i.p. injection of 90 mg/kg-bw ketamine hydrochloride (Ketalar, Eczacıbaşı İlaç A.Ş., Istanbul, Turkey) and 10 mg/kg-bw xylazine (Alfazyne, Alfasan International B.V., Woegen, Holland). The hair coat of the dorsal area was removed with an electrical shaver and the skin was disinfected with 70% alcohol solution. Six circular full-thickness skin wounds (= 8 mm) were created using a sterile biopsy punch (Sevimli-Gur et al., 2011).

Everyday, test materials (Six full-thickness skin defects were made on the back of the rats; 1: Placebo gel; 2: 0.5% PSE; 3: 2.5% PSE; 4: 1.25% PSE; 5: 2.5% C-PC; 6: 1.25% C-PC. Every day, the rats were examined, the length and width of the lesions were measured. To evaluate the efficiency of the materials, specimens encompassing the whole area were removed under general anesthesia on 3rd, 7th and 14th days after the operation. Specimens were fixed in formaldehyde for histological examination) were applied to the wounds on each animal. Each wound was considered as one group (Figure 1).

Histological examination

The wound specimens including full thickness skin layers (epidermis, dermis, and hypodermis) and the underlying muscle layer were fixed in 10% buffered formaldehyde and processed according to the routine light microscope tissue processing methods. Processed tissues were embedded in paraffin. 5 μm tissue sections stained with HE were examined and photographed



Figure 1. Groups of the test materials. Six full-thickness skin defects were made on the back of the rats. 1: Placebo gel; 2: 0.5% PSE; 3: 2.5% PSE; 4: 1.25% PSE; 5: 2.5% C-PC; 6: 1.25% C-PC. Every day, the rats were examined; the length and width of the lesions were measured. To evaluate the efficiency of the materials, specimens encompassing the whole area were removed under general anesthesia on 3rd, 7th and 14th days after the operation. Specimens were fixed in formaldehyde for histological examination.

Table 1. The scoring system using histological examination.

Criteria	Score			
	0	1	2	3
Reepithelization	None	Partial	Complete, but immature or thin	Complete and mature
Neovascularization	None	Up to 5 vessels / HMF	6-10 vessels / HMF	>10 vessels / HMF
Granulation tissue amount	None	Scant	Moderate	Abundant
Granulation tissue maturation	Immature	Mild maturation	Moderate maturation	Fully matured
Inflammatory cells	None	Scant	Moderate	Abundant

by Leica image analyzing system (Leica, Germany). All specimens were evaluated individually by two histologists who were blinded to the test material type and the time from wounding. The main histological criteria were reepithelization, neovascularization, presence of inflammatory cells, granulation tissue amount and maturation (Table 1). The scoring system used was modified from the one suggested by Abramov et al. (2007).

Statistical analysis

The resulting data were subjected to two-tailed paired t-test for statistical significance. p value < 0.05 was considered significant.

RESULTS

In vitro wound healing and keratinocyte proliferation, migration activities

Cell viability and proliferation effects of the samples and the positive control were analyzed by MTT assay at 570 to 690 nm. For assessment of *in vitro* wound healing

effect of the samples in terms of cell viability and proliferation, human keratinocytes were treated for 72 h with tenfold dilutions of samples ranging from 33.5 to 0.0335 $\mu\text{g/mL}$ and EGF (10 ng/mL) as a positive control (Figure 2). PSE and C-PC showed a similar proliferation profile in terms of cell viability percentage where an increase in cell proliferation was observed at the end of 72 h of treatment. Compared to both the negative and the positive controls, EGF (10 ng/mL), PSE and C-PC showed stronger and more significant proliferation activities of 270 and 213% cell viability, respectively with 33.5 $\mu\text{g/mL}$ doses on keratinocytes. Moreover, cell viability ranged from 205 to 270% in the PSE and from 117 to 213% in the C-PC with the doses ranging from 0.0335 to 33.5 $\mu\text{g/mL}$. Cell viability and proliferation difference between PSE and C-PC was not significant ($p > 0.05$) at the range of studied doses. Cell viability of the rest of the EGF (10 ng/mL) group showed a similar increasing trend from 100 to 165% at the end of 72 h.

Number of the cells formed as a result of migration and proliferation was determined with a random measurement

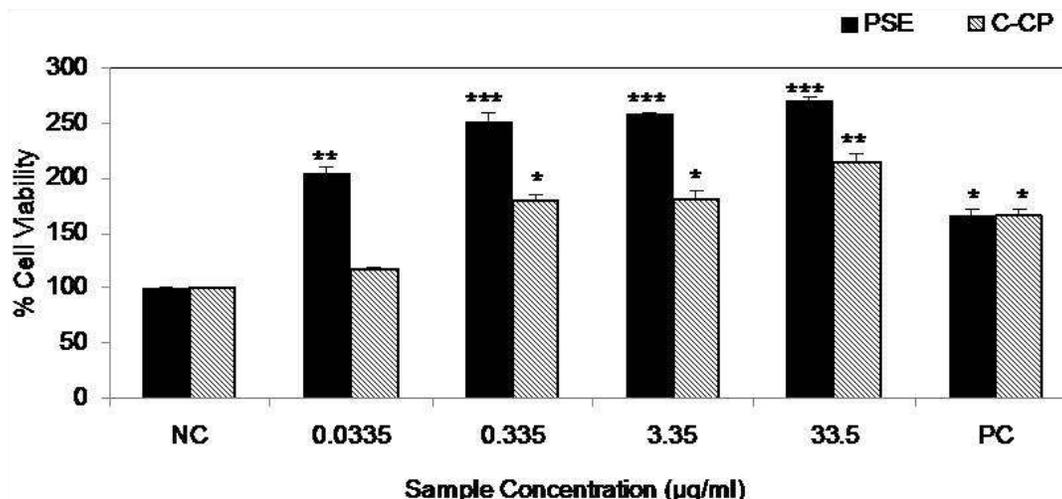


Figure 2. Cell viability and proliferation effect of PSE and C-PC, results of MTT assay. To assess the effect of PSE and C-PC on exponentially growing human keratinocytes, 6,000 cells/well were plated in 96 well/plates and the next day, cells were treated with either DPBS vehicle control or PSE and C-PC samples ranging from 33.5 to 0.0335 µg/mL doses or positive control EGF (10 ng/mL), all in complete medium. After 72 h of these treatments, cell viability and proliferation were determined. The data were obtained from three independent assays using three wells for each assay (mean ± SE). "NC" [cell + medium + DPBS] denotes negative control whereas "PC" stands for positive control (EGF, 10 ng/mL). The resulting data were subjected to two-tailed paired t-test for statistical significance * $p < 0.04$, ** $p < 0.01$, *** $p < 0.001$ versus the control.

of quantity of cells (number of cells) in the wound site (Figures 3 and 4). Proliferation, healing and migration increased in a dose dependent manner both in C-PC and in PSE at doses ranging from 0.0335 to 33.5 µg/mL (Figures 3 and 4). PSE and C-PC showed the best growth stimulation effect with 33.5 µg/mL dose which increased the quantity of cells measured from 267 ± 2 to 437 ± 14 and from 267 ± 5 to 380 ± 11 , respectively at the end of 72 h of treatment (Figures 3 and 4). As for EGF, quantity of cells obtained for the positive control was measured as 332 ± 8 as a result of the same treatment (Figures 3 and 4). PSE and C-PC showed similar proliferative profiles with the MTT assay given above in terms of cell viability and quantity of the cells. The quantity of cells measured increased from 372 ± 6 to 437 ± 7 for PSE and from 284 ± 3 to 380 ± 6 for C-PC at doses ranging from 0.0335 to 33.5 µg/mL at the end of 72 h (Figures 3 and 4). Differences in proliferation between PSE and C-PC were found not to be significant ($p > 0.05$) for all of the applied doses.

***In vivo* tissue regeneration activities**

In all placebo gel applied groups, the wound region consisted of epidermis, dermis, subcutaneous fat and muscle tissues. After 3 days in the wound region, epithelium was missing under the crust. Between the edematous collagen fibers of the connective tissue, mononuclear cell infiltration was evident. The blood

vessels were dilated with stasis and extending through the depth of the wound. In 7 days, epithelization has started at the ends of the wound region but it was missing on the surface which was covered by granulation tissue and crust. A large scar tissue was extending all through the dermis. Muscle layer was missing under the scar tissue but at the junction of the scar and the healthy skin, muscle bundles were observed to be pulled towards the scar by collagen fibers filling the region under the scar. In 14 days, the wound was completely healed, epithelization was completed, hair follicles regenerated and reached to the surface. The ends of the muscle layers on each side were joined by a thick and dense connective tissue layer containing newly formed muscle fibers.

In all 0.5% PSE applied groups, the wound was extending deep to the muscle layer. In 3 days, the surface was covered with crust in 4 of the animals. Under the crust, there was a thick vascular granulation tissue in the dermis above the hypodermis rich in fat cells. The epithelium was absent and the muscle layer below the hypodermis was discontinuous. In the 7th day, a thin continuous epithelium was covering the surface of dense connective tissue rich in blood vessels. There was a deposition of collagen and fibroblasts in the wound region filling the dermis region. The muscle layer below this region was still missing. At the borders of the scar tissue, edematous collagen fibers were observed with scattered infiltrative cells. In the 14th day, the surface was completely covered by the epithelium. The collagen fibers of the dermis were almost normal with some hair follicles

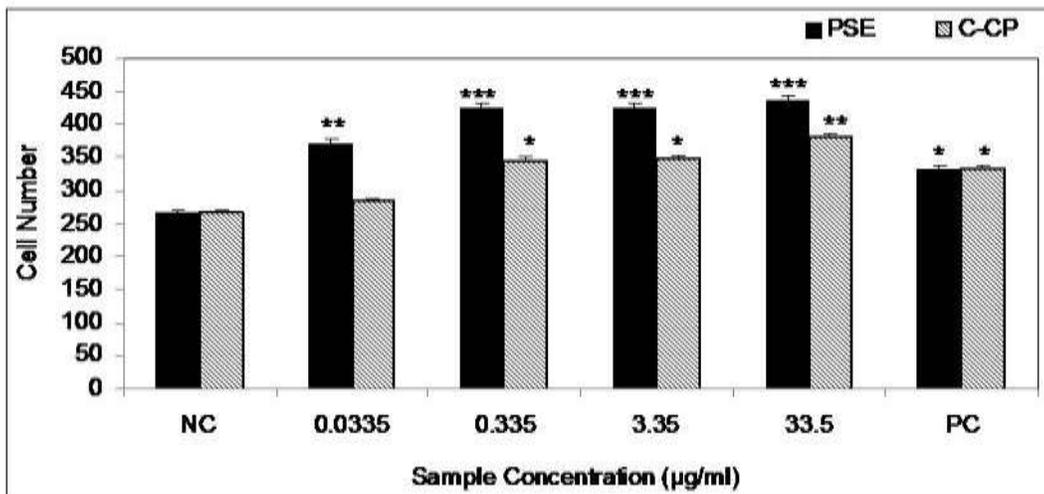


Figure 3. Wound healing, cell proliferation and migration effects of crude PSE and C - PC obtained from in vitro wound healing study. To assess the proliferation, migration and wound healing effects of PSE and C - PC on human keratinocytes, 200.000 cells/well were plated in 24 - well/plates and they were cultured covering the surface completely. By using a sterile teflon bar that removes cells, a wound was formed by scratching in a 5 mm diameter circular zone of cell layer. As negative control groups (NC) DMEM - HAM's / F - 12 medium with 10 % FCS, DMEM - HAM's / F - 12 medium without 10 % FCS and HBSS and as the positive control group (PC) 10 ng/mL of EGF were used. At the end of 72 hours of incubation, cells were fixed with 4 % paraformaldehyde and stained with Giemsa and / or HE. Healing in the wound zone was photographed. The number of cells formed as a result of migration and proliferation was determined after transferring the pictures to a computer. The data were obtained from three independent assays using three wells for each assay (mean ± SE). The resulting data were subjected to two - tailed paired t - test for statistical significance. *p < 0.04, **p < 0.01, ***p < 0.001 versus the control.

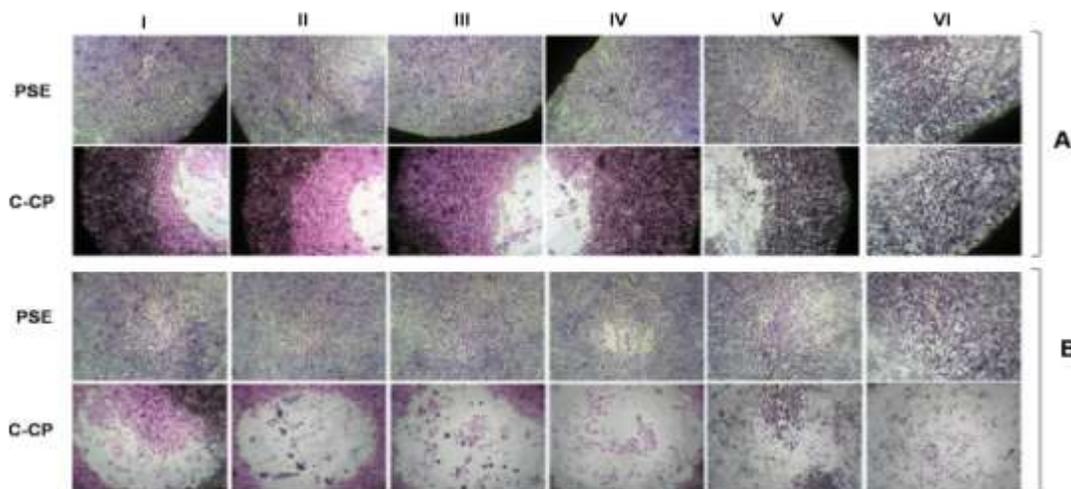


Figure 4. Morphological observation of the wounded edge and centre of HS2 cell cultures. Confluent cultures of the cells were wounded and incubated for 72 h in presence of PSE and C - PC at doses ranging from 33.5, 3.35, 0.335, 0.0335 µg/mL. I. 33.5 µg/mL; II. 3.35 µg/mL; III. 0.335 µg/mL; IV. 0.0335 µg/mL; V. Negative control (DMEM - HAM' s / F - 12); VI. positive control (EGF, 10 ng/mL). PSE denotes crude Spirulina extract whereas "C - PC" stands for C - phycocyanin. A. wound edge; B. centre of wound (X40).

extending to the surface, the muscle layer was still discontinuous. Collagen fibers filled the spaces between muscle bundles.

In 3 days of 1.25% PSE group, the epithelium was

missing under the crust layer. The wound region was filled with granulation tissue rich in infiltrative cells. The muscle layer below was discontinuous. In the 7th day group, the crust was still persisting in three of the

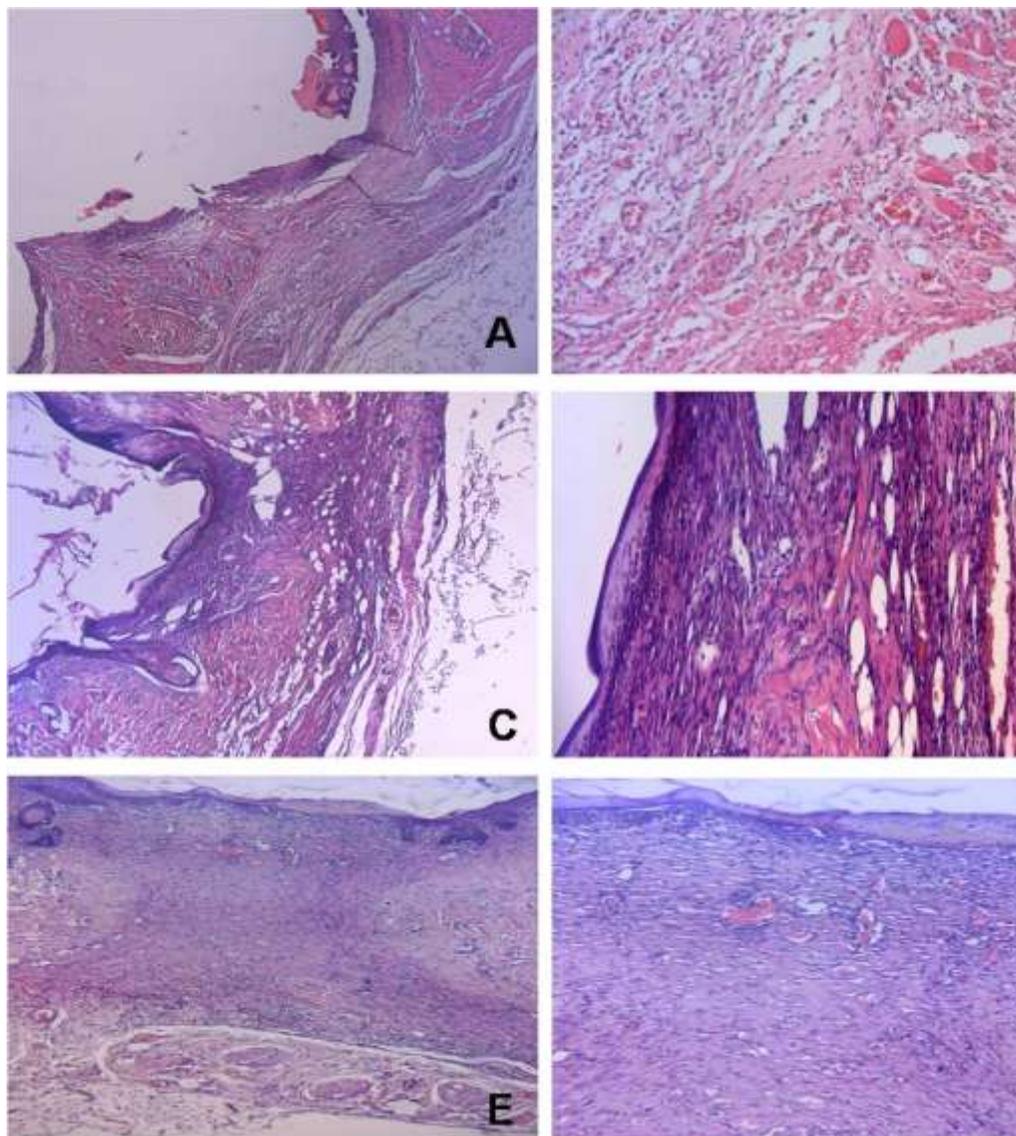


Figure 5. 1.25 % C - PC group; granulation tissue with dilated blood vessels filled the wound region in 3 days A: (HE X100), B: (HE X200). In 7 days an immature epithelium covered the surface. C: (HE X100), D: (HE X200). In 14 days a very thick and dense scar tissue was formed E: (HE X100), F: (HE X200).

animals. The wound surface was covered by a thin epithelium; the underlying scar tissue was rich in blood vessels. There was a rather loose connective tissue below the scar. The muscle layer was still discontinuous. In the 14th day, the size of the scar tissue was highly diminished, the dermis except the superficial and deeper regions resembled normal dermis structure but the region of the muscular tissue was filled with a connective tissue.

In 2.5% PSE group, in the 3rd day samples, the structure of the wound region was the same as the other groups. However, in the 7th day group, the epithelization started at the sides of the wound region but the center was not covered by the epithelium. A thick scar tissue rich in fibroblasts and blood vessels was extending to the

surface, the region of the striated muscles was filled with a rather loose connective tissue containing blood vessels. On the 14th day the epithelium was completely covering the wound surface but the collagen fibers filling the dermal region was edematous and the newly formed scar tissue was extremely dense and thick. The region in between the muscle fibers was filled with a rather loose connective tissue containing dilated blood vessels.

In 1.25% C-PC group, on the 3rd day, the structure was the same as the 3rd day of the previous groups (Figure 5a and b). The epithelium over the scar tissue which filled the wound region was recovered on the 7th day. The muscle layer was still missing but its place was filled with dense connective tissue (Figure 5c and d). On the 14th

day, a rather thin epithelium was observed to cover the surface. A very thick and dense scar tissue was formed above the muscle bundles which are newly formed (Figure 5e and f). It was observed that 1.25% C-PC had a better effect on the 7th day compared to other test materials but on the 14th day, both the epithelium and the scar tissue development were worse than the others.

On the 3rd day of 2.5% C-PC treatment, the wound region was still covered by the crust and the other regions resembled the 3rd day of the previous groups. On the 7th day, the epithelium was still missing in the center of the wound but it started to proliferate at the borders. A thick scar tissue was developed, extending deeply towards the region of the dense connective tissue which filled the missing muscle layer. On the 14th day, five of the animals had an almost completely healed wound region whereas in two of them, newly formed collagen bundles were coarser and edematous.

DISCUSSION

Wound healing is a fundamental response to tissue injury that involves a complex set of cellular, physiological, and molecular events targeted toward the restoration of the structural and functional integrity of the damaged tissue (Raf, 1996; Priya et al., 2004). Many plant-based products have been shown to have therapeutic potential as promoters of wound healing (Priya et al., 2004; Paiva et al., 2002). Earlier reported anti-inflammatory (Singh et al., 2005; Plaza et al., 2009; Abed et al., 2009), antioxidants (Singh et al., 2005; Plaza et al., 2009; Abed et al., 2009) and anti-microbial activity (Singh et al., 2005; Plaza et al., 2009; Hayashi et al., 1996; Abed et al., 2009) of PSE and C-PC (Farooq et al., 2004; Kim et al., 1998; Kaji et al., 2002; Qishen et al., 1988; Reddy et al., 2003; Li et al., 2005; Patel et al., 2005; Abd El-Baky et al., 2009) satisfied the basic criteria for being used in wound management. Moreover, *Spirulina* may have protective effect against cell damage caused by radiation (Samarth et al., 2008; Klingler et al., 2002; Belay et al., 1993). A survey of the literature revealed that there was no wound healing study on PSE and C-PC. Herein, we found that the PSE and C-PC extract directly enhances wound repair.

The chemical basis of the promotion of healing is the subject of further investigation. At present, it is tempting to speculate that the minerals, phycobiliproteins, vitamins, beta carotene, fatty acids, polysaccharides, phenolic compounds and volatile compounds present in these microalgae may accelerate wound healing by acting as an anti-oxidant and scavenging destructive free radicals responsible for cell death (Kim et al., 1998; Subhashini et al., 2004; Qishen et al., 1988; Reddy et al., 2003; Li et al., 2005; Patel et al., 2005; Hirahashi et al., 2002; Abd El-Baky et al., 2009; Singh et al., 2005; Plaza et al., 2009). These reports confirmed that some unidentified factors in the crude extract might have enhanced the

healing process. Stimulation of keratinocyte is one mechanism by which PSE might enhance wound repair process. But Ca-SP which is a sulfated polysaccharide isolated from PSE may retard the repair process of damaged vascular endothelium through inhibition of vascular endothelial cell proliferation by induction of a lower ability to respond to stimulation by endogenous basic fibroblast growth factor (Kaji et al., 2002).

Conclusion

This study confirms a promising wound healing activity of PSE in wounds as shown by *in vivo* data, as well as normal wound healing responses *in vitro*. Proliferation and growth stimulation activities of PSE seem to be directly associated with either C-PC or potentially other unidentified compounds (Kim et al., 1998; Subhashini et al., 2004; Qishen et al., 1988; Reddy et al., 2003; Li et al., 2005; Patel et al., 2005; Hirahashi et al., 2002; Abd El-Baky et al., 2009; Singh et al., 2005; Plaza et al., 2009; Hayashi et al., 1996; Abed et al., 2009). Based on the results of this study, it can be suggested that new proliferative molecules should be found by bioactivity guided isolation of PSE in the following studies. Slight differences were obtained with the PSE and C-PC. On the other hand, PSE contains a mixture of proteins and carotenoids which interact synergistically in mediating proliferation of skin cells and hence contribute significantly to wound healing and tissue regeneration (Kim et al., 1998; Subhashini et al., 2004; Qishen et al., 1988; Reddy et al., 2003; Li et al., 2005; Patel et al., 2005; Hirahashi et al., 2002; Abd El-Baky et al., 2009; Singh et al., 2005; Plaza et al., 2009; Abed et al., 2009). Further studies are warranted to understand the mechanism of action of remarkable wound healing extract and agent C-PC.

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