

Original Article	The Effect of Spirulina Extract on the Healing of Skin Wounds in Adult Albino Rats: A Light and Scanning Electron Microscopic Study <i>Ibtisam Ahmed Bahei-Eldin, Mona Hamed Elgazzar, Asmaa Ibrahim Ahmed, Shimaa Saeed Attia Mohamed</i> <i>Anatomy and Embryology Department, Faculty of Medicine-Ain Shams University</i>
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ABSTRACT

Aim of the Work: Wounds are common in clinical practice. Spirulina is analga, consumed as a dietary supplement and it is well known as apotent antioxidant. Therefore, the aim of this study was to determine the effect of topical application of spirulina extract on the healing process in a rat model of excisional skin wound.

Material and Methods: Seventy two adult male albino rats weighing 180200- g were used. An excisional skin wound of 2 cm diameter was performed on their mid-back. The animals were divided into four groups (18 rats per group): group I (untreated control group), group II (xanthan gum treated group); this gum was used as vehicle for spirulina. Group III(carboxymethyl cellulose (CMC) treated group) which was the reference group and group IV (spirulina treated group). In II, III and IV groups the material (0.2ml) was topically applied twice daily. The rats of all groups were sacrificed on day 3, 7 and 12 post-wounding (6 rats per day).

Specimens from the site of the wound and 2 mm of adjacent normal skin were collected. Some of them were fixed in 10% neutral formalin for preparation of paraffin sections which were stained with hematoxylin and eosin and Mallory's trichrome. Other specimens were fixed immediately in 2.5% glutaraldehyde and processed for scanning electron microscopic examination (SEM). Morphometric study was performed using image analyzer for the following parameters: counting the number of newly formed blood vessels, macrophages and fibroblasts and also calculating area % of collagen fibers deposition. Statistical analysis was done using ANOVA test.

Results: The histological findings of xanthan gum group were similar to those of the control group on day 3, 7 and 12 post-wounding. On day 3 post-wounding the epidermis at the wound area was absent in all groups, but spirulina treated group showed beginning of re-epithelialization. Few inflammatory cells were detected in the granulation tissue in the spirulina treated group while heavy inflammatory infiltrate was observed in control and CMC treated groups. On day 7 post-wounding the epidermis of the spirulina treated group showed mature differentiation and was regularly arranged in four layers unlike the immature differentiation of other groups. On day 12 post-wounding the spirulina treated group showed that the wound area was bridged by the newly formed thick epidermis with complete re-epithelialization and appearance of skin appendages (hair follicles) in contrary to other groups. In the granulation tissue the image analysis for the spirulina treated group showed a highly statistical significant increase in the number of newly formed blood vessels, and fibroblast on day 3 and 7 post-wounding and decrease on day 12 post-wounding. The macrophages count showed highly statistical significant increase in spirulina treated group only on day 3 post-wounding. In spirulina treated group, Mallory's trichrome stained sections on day 7 and 12 post-wounding revealed regular collagen fibers aligned horizontally with high statistically significant increase in area % of collagen fibers deposition. SEM revealed that the collagen fibers were discriminated as three zones (sub-epithelial, middle and deep) in spirulina treated group on day 7 and 12 post-wounding. These zones were only observed on day 12 post-wounding in control and CMC treated groups.

Conclusion: Spirulina extract significantly promoted the rate of skin wound closure. It enhanced re-epithelialization and improved the neovascularization process at the wound site. It also augmented fibroblast proliferation and collagen synthesis as well as proliferation of hair follicles; in contrast to CMC treated group and the control group.

Key Words: Excisional Skin Wound, Spirulina Extract Preparation, Adult Albino Rat, Light Microscopy, Scanning Electron Microscopy.

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INTRODUCTION

The skin's ability to heal wounds quickly and effectively is essential for good health (Morton and Phillips, 2016). Wound healing is a complex and dynamic cascade that has attracted the attention of researchers for many years. It is a process essential for the restoration of the integrity and function of the damaged tissue as closely as possible to its normal state. Normal wound healing response begins the moment the tissue is injured. It includes several chemical mediators and growth factors and involves continuous cell-cell and cell-matrix interactions that allow the process to proceed in three overlapping phases, inflammation, proliferation and remodeling (Rieger et al., 2014). In developing countries, wounds constitute a major problem as the rate of severe complications is high and financial resources are limited. This increases the popularity of natural therapy which represents the hope of finding a product with both higher efficacy and less cost for wound healing (Gorski and Novella, 2014).

Spirulina is a blue-green alga (cyanobacterium) that has been consumed as food since ancient times. The alga is presently marketed as a food supplement (nutraceutical) due to its high contents of proteins, α -linolenic acid, vitamins and minerals (Wang et al., 2008).

Recent studies have demonstrated the antioxidant, antimutagenic, antiviral, anticancer, antiallergic, immune enhancing, hepato-protective, blood vessel relaxing and hypolipidemic effects of spirulina extracts (Gershwin and Belay, 2008; Mani et al., 2008; Mala et al., 2009).

Its therapeutic implications in cases of diabetes, arthritis, anemia, cardiovascular diseases and cancer were reported (Soheili and Khosravi-Darani, 2011; Hoseini et al., 2013). Reviewing the literature, few studies were met with concerning the use of spirulina extract in skin wound healing (Madhyastha et al., 2012; Gur et al., 2013). The present study was carried out to determine the effect of the spirulina extract on the healing process of skin wounds in adult male albino rats using light and scanning electron microscope.

MATERIAL AND METHODS

Experimental animals:

In the present study 72 adult male albino rats weighing 180 -200 g were obtained from the animal house, Faculty of Medicine, Ain Shams University. Animals were maintained according to the principles and guidelines of the Committee of Animal Research Ethics (CARE). They were housed in stainless steel cages, two rats per cage, and were left for a week before any intervention to acclimate to experimental conditions. The rats were exposed to 12 hours light/dark cycle and allowed daily diet and free water access (*ad libitum*) with suitable environmental conditions and good ventilation.

Materials used in the present study included the following:

1. Spirulina (*Arthrospira platensis*):

It was in the form of dark blue-green dry powder, purchased from Source Naturals, Scotts valley, CA, US.

Spirulina extract preparation:

Spirulina extract was prepared in the Biochemistry Department laboratory, Faculty of Medicine, Ain shams University according to the method of Syarina et al. (2015). Ten grams of dried spirulina were mixed to 500 ml of ultrapure water for 24 h at room temperature, then the mixture was centrifuged at 3000 rpm for 10 min (4°C) and the supernatant was filtered (whatman No.1) to remove cell debris. The samples were then evaporated at 40°C by vacuum rotatory evaporator. The dried extract was then kept at 4°C until further use.

Xanthan gum:

It was in the form of white dry powder, purchased from Sigma chemical company (Cairo, Egypt). It was used as a vehicle for the topical preparation of spirulina extract (Mahmood et al., 2010). 200 mg of xanthan gum in 10 ml normal saline were used for dissolving 3 grams of spirulina extract, so one ml contained 300 mg which was the maximum tolerated dose

per kg b. w. (Panigrahi *et al.* 2011). From this preparation 0.2 ml was used topically twice daily for the wound of each rat.

2. Carboxymethyl cellulose (CMC):

It was in the form of white dry powder, purchased from Sigma chemical company (Cairo, Egypt). It was used to prepare a colorless transparent aqueous gel, which was formed of 2.3% of carboxymethyl cellulose polymer together with propylene glycol (20%). Then, 0.2 ml of CMC gel was applied topically twice daily to the wounds of rat's skin (Mughrabi *et al.*, 2014).

Experimental design:

An excisional skin wound (2 cm in diameter) was performed in the mid-back of the rats (Fig. A). The 72 animals were divided into the following four groups: I, II, III, and IV (18 rats each). Then each group was divided into 3 sub-groups (6 rats each), accordingly skin wound specimens were examined on days 3, 7 and 12 post-wounding.

Group I (control group): The rats with skin wound were kept untreated.

Group II (xanthan gum treated group): The rats with skin wound were topically treated twice daily with 0.2 ml of xanthan gum in normal saline (20 mg/ml).

Group III (CMC treated group): The rats of this group were served as reference group. Their skin wounds were topically treated twice daily with 0.2 ml of CMC gel.

Group IV (spirulina treated group): The rats with skin wound were topically treated twice daily with 0.2 ml of spirulina extract preparation in the vehicle (xanthan gum) (Fig. B).

Preparation of excisional skin wound:

The animals were anesthetized with an intramuscular injection of 7 % ketamine solution and 0.3 % xylene solution at a ratio of 2:1 (0.2 mL/100 g b. w.) (Fathke *et al.*, 2004). All care was taken to avoid any discomfort to the animals. Once anesthetized, the animals were placed in the prone position, the wound site was sterilized with an alcohol-iodine solution, and they were held

while the dorsum was shaved (De Melo Rambo *et al.*, 2014).

A uniform circular area 2.00 cm in diameter was excised from the skin using a circular stamp and sterile scissor. The wounds were located in the middle portion of the median sagittal plane of the back of each animal and left uncovered. After recovery, animals were returned to their housing and allowed chow and water; everyone in a separate cage (Hajiaghaalipour *et al.*, 2013).

Collection of specimens:

The rats were sacrificed on days 3, 7 and 12 post-wounding by decapitation under anesthesia by ether inhalation. The entire wound area including the adjacent 2 mm of normal skin margins were excised. Some of specimens were fixed in 10% neutral formalin for 48 hours and processed for light microscopic examination, while other specimens were fixed immediately in 2.5% glutaraldehyde and processed for scanning electron microscopic (SEM) examination.

Tissue processing for light microscopic study:

Skin wound specimens, fixed in 10% neutral formalin, were prepared for paraffin blocks. Serial sections from paraffin blocks of 5 µm thickness were cut perpendicular to the surface of the wound. Some sections were stained with hematoxylin and eosin (Hx&E) while other sections were stained with Mallory's trichrome for demonstrating the collagen fibers (Drury and Wallington, 1980).

Tissue processing for scanning electron microscopic study:

Skin wound specimens, fixed in 2.5% glutaraldehyde, were dehydrated in ascending grades of ethanol and then in 100% acetone. Specimens were then cut by a sharp blade razor at the site of the wound and examined on their side i.e. perpendicular to the surface to allow examination of all layers of the skin. The specimens were then dried at critical point using liquid carbon dioxide in (Tousimis Audosamdri-815), fixed on aluminum stubs exposing the cut surface for examination and then sputter coated with gold using (SPI-Module) (Robinson *et al.*, 1987). They were examined with scanning electron microscope (JEOL-JSM-5500LV) by using high vacuum

mode at the Regional Center of Mycology and Biotechnology, AL Azhar University.

Computer image analysis:

Morphometric study was performed using image analyzer Leica (Q 500 MC program, Wetzlar, Germany) in the Anatomy Department, Faculty of Medicine, Ain Shams University. Data were collected by examining 5 different fields in 5 different sections from the six animals in each group at X10 low power field for blood vessels count and area percent for collagen fibers deposition and at X40 high power field for fibroblasts and macrophages count.

Statistical analysis:

Statistical analysis was done using the SPSS software (Statistical Package for Social Studies-version 13.0). One way analysis of variance (ANOVA) was employed to compare means in different groups with each other. Bonferroni Post Hoc test was used to detect significance between every two individual groups.

The significance of the data was determined by the probability (P. value). $P > 0.05$ was considered non-significant. $P \leq 0.05$ was considered significant and $P \leq 0.001$ was considered highly significant. Data were represented in tables and histograms, prepared by using MS Excel 2013.

RESULTS

I. Histological Results:

In the present study, the histological findings of skin wound of the rats of group I (control group) and of the rats of group II (Xanthan gum treated group) showed similar results on day 3, 7 and 12 post-wounding.

Examination of hematoxylin and eosin stained skin sections of the wound of the control group I (day 3 post-wounding) revealed the presence of a thin scab covering the wound area (Fig. 1). Beneath the scab, the epidermis was absent and the granulation tissue was observed filling the wound gap (Fig. 1). The granulation tissue was formed of a combination of cellular elements, numerous newly formed small blood vessels (angiogenesis) embedded in loose extracellular matrix of collagen. The cellular elements of the granulation tissue included polymorphnuclear leucocytes,

lymphocytes, macrophages and fibroblasts. The macrophages exhibited a large indented nucleus. Extravasation of red blood cells was evident in the granulation tissue (Fig. 2).

In Mallory's trichrome stained sections, few dispersed interrupted thin collagen fibers were demonstrated in the granulation tissue (Fig. 3).

Examination of hematoxylin and eosin stained skin sections of the wound of CMC group III (day 3 post-wounding) revealed the presence of a thin scab covering the wound area (Fig. 4). The epidermis was absent at the wound area and the granulation tissue showed newly formed blood vessels (Fig. 4). Few fibroblasts, polymorphnuclear cells and many macrophages were also noted (Fig. 5).

In Mallory's trichrome stained sections, few scattered thin collagen fibers in the granulation tissue were observed (Fig.6).

Examination of hematoxylin and eosin stained skin sections of the wound of spirulina treated group IV (day 3 post-wounding) revealed the presence of a thick scab covering the wound area. Beneath the scab there was an early growth of thickened epidermis over the edges of the wound i.e. beginning of re-epithelialization (Fig. 7). The newly formed granulation tissue proliferated to the same level of the adjacent nearby skin edges filling the wound area completely with areas of angiogenesis (Fig. 7). The granulation tissue showed numerous newly formed blood vessels, fibroblasts and macrophages (Figs. 7, 8).

In Mallory's trichrome stained sections, early deposition of newly formed thin deeply stained irregularly distributed collagen fibers was observed in the granulation tissue (Fig. 9).

Examination of hematoxylin and eosin stained skin sections of the wound of control group I (day 7 post-wounding) revealed the presence of a thick scab covering the wound area (Fig. 10). Beneath the scab there was evident growth of epidermis over the edges of the wound i.e. re-epithelialization. The newly formed epidermis showed immature differentiation (Fig. 10). The underlying granulation tissue showed persistence of inflammation with occurrence of many polymorphnuclear cells (Fig. 11). Also numerous

blood vessels, fibroblasts and macrophages were observed (Figs. 10, 11).

In Mallory's trichrome stained sections, few collagen fibers were observed in the granulation tissue. They were haphazardly arranged (Fig. 12).

Using scanning electron-microscope, the skin wound showed the newly formed granulation tissue that contained few scattered extensively irregular collagen fibers separated by wide spaces (Fig. 13).

Examination of hematoxylin and eosin stained skin sections of the wound of CMC group III (day 7 post-wounding) revealed poorly differentiated epidermis over the edges of the wound i.e. re-epithelialization (Fig. 14). The underlying granulation tissue showed inflammatory cell infiltrate, macrophages and numerous blood vessels. Fibroblasts were also frequently observed (Fig. 15).

In Mallory's trichrome stained sections, many scattered collagen fibers were noted at the site of the wound (Fig. 16).

Using scanning electron-microscope, the skin wound showed that the re-epithelialization was covering the wound area. The underlying granulation tissue was formed of few wide spaced collagen fibers (Fig. 17).

Examination of hematoxylin and eosin stained skin sections of the wound of spirulina treated group IV (day 7 post-wounding) revealed newly formed epidermis beneath the scab. The epidermis showed mature differentiation and it was regularly arranged in four layers (Fig. 18, A). Stratum basale was formed of crowded low columnar basophilic cells with basal oval nuclei. Superficial to this layer, prickle cell layer consisted of few polyhedral acidophilic cells with central rounded nuclei. Cells of stratum granulosum were flattened with basophilic granular cytoplasm. Finally, the stratum corneum appeared to be formed of few acidophilic scales (Fig. 18, B). The underlying granulation tissue showed few inflammatory cell infiltrate, many blood vessels and fibroblasts with few macrophages (Fig. 19).

In Mallory's trichrome stained sections, collagen fibers were aligned parallel to the surface.

They were deposited in between and around the blood vessels (Fig. 20).

Using scanning electron-microscope, the skin wound showed that the granulation tissue was discriminated into 3 zones; sub-epithelial zone, middle zone and deep zone. The sub-epithelial zone was formed of thin collagen fibers arranged haphazardly beneath the epidermis. The middle zone was formed of collagen fibers deposited around the blood vessels. The deep zone was formed of collagen fibers arranged parallel to the surface (Fig. 21).

Examination of hematoxylin and eosin stained skin sections of the wound of control group I (day 12 post-wounding) revealed complete absence of the scab. The newly formed thick epidermis extended from the wound edges towards its center with incomplete re-epithelialization. The underlying granulation tissue showed numerous blood vessels and collagen fibers deposition (Fig. 22). Also many macrophages and fibroblasts were observed with few polymorphonuclear cells (Fig. 23).

In Mallory's trichrome stained sections, collagen fibers were aligned horizontally in between the blood vessels (Fig. 23).

Using scanning electron-microscope, the granulation tissue at the wound area is discriminated into 3 zones, sub-epithelial, middle and deep zone. The newly formed epidermis (E) is separated from the sub-epithelial zone (Fig. 24).

Examination of hematoxylin and eosin stained skin sections of the wound CMC group III (day 12 post-wounding) revealed complete absence of the scab. The newly formed thick epidermis extended from the wound edges towards its center with incomplete re-epithelialization (Fig. 25). The underlying granulation tissue showed few inflammatory cell infiltrate, macrophages, fibroblasts and numerous blood vessels. In Mallory's trichrome stained sections; collagen fibers were aligned horizontally (Fig. 26).

Using scanning electron-microscope, the skin wound showed that the newly formed epidermis extended to cover the wound area. The

underling granulation tissue is discriminated into 3 zones, sub-epithelial zone, middle zone, deep zone (Fig. 27).

Examination of hematoxylin and eosin stained skin sections of the wound of the spirulina treated group IV (day 12 post-wounding) revealed complete absence of the scab. The wound area was bridged by the newly formed thick epidermis showing complete re-epithelialization. Evident reduction in the wound gap was noticed (Fig. 28).

Mallory's trichrome stained sections revealed dense deposition of deeply stained regularly arranged collagen fibers with intervening blood vessels in the regenerated dermis (Fig. 29).

Using scanning electron-microscope, the skin wound appeared completely covered by the epidermis. The underlying regenerated dermis was discriminated into 3 clear zones;

sub-epithelial, middle and deep zones. The sub-epithelial zone was formed of regularly arranged thin collagen fibers adherent to the epidermis (Fig. 30). Appearance of skin appendages (hair follicles) was also noted (Fig. 31).

(SEM X250)

II. Morphometric Results:

In the present study, skin wounds of the rats of group II (xanthan gum treated group) showed statistical non-significant difference in the number of blood vessels, macrophage count, fibroblast count and area percent of collagen fibers deposition on day 3, 7 and 12 post-wounding compared to the control group. Mean \pm SD were expressed in tables (1 -4) and in histograms (1 -4).

- $P > 0.05$ was considered non-significant.
- $P \leq 0.05$ was considered significant.
- $P \leq 0.001$ was considered highly significant.

1- Blood vessels count: Table (1) and in histogram (1).

2- Table 1: Number of blood vessels / high power field (x100).

<i>P</i> value	Mean	\pm SD	Groups	Post-wounding days
	14.83	\pm 4.26	Control group	Day 3
1.000	16.17	\pm 3.06	Xanthan treated group	
0.210	21.33	\pm 2.42	CMC treated group	
0.000**	29.67	\pm 3.39	Spirulina treated group	Day 7
	22.21	\pm 4.47	Control group	
0.347	22.83	\pm 5.38	Xanthan treated group	
1.000	28.17	\pm 2.64	CMC treated group	Day 12
0.000**	40	\pm 2.61	Spirulina treated group	
	24.5	\pm 4.64	Control group	
1.000	23.67	\pm 3.93	Xanthan treated group	Day 12
0.445	18.5	\pm 4.04	CMC treated group	
0.001**	14.67	\pm 3.56	Spirulina treated group	

**The number of new blood vessels is highly significant increased ($P \leq 0.001$) on day3 and 7 post-wounding and decreased on day 12 post-wounding compared to the control.

2- Macrophage count: Table (2) and in histogram (2).

Table 2: Number of macrophages / high power field(x400)

P value	Mean	±SD	Groups	Post-wounding days
	7	±2.45	Control group	
1.000	7.62	±2.19	Xanthan treated group	Day 3
0.000**	19.17	±3.31	CMC treated group	
0.000**	23.5	±6.06	Spirulina treated group	
	12.67	±3.50	Control group	
1.000	12.85	±3.67	Xanthan treated group	Day 7
1.000	17	±2.19	CMC treated group	
0.474	18.17	±4.79	Spirulina treated group	
	13.5	±3.08	Control group	
1.000	12.17	±3.92	Xanthan treated group	Day 12
1.000	9.67	±2.16	CMC treated group	
0.616	8.17	±1.72	Spirulina treated group	

The number of macrophages is highly statistical significant increased on day 3 post- wounding compared to the control group.

3- Fibroblast count: Table (3) and in histogram (3).

Table 3: Number of fibroblasts / high power field(x 400)

P value	Mean	±SD	Groups	Post-wounding days
	2.5	±1.05	Control group	
1.000	2.71	±1.51	Xanthan treated group	Day 3
1.000	5.67	±1.86	CMC treated group	
0.000**	27.33	±1.97	Spirulina treated group	
	12.5	±2.51	Control group	
1.000	12.84	±1.897	Xanthan treated group	Day 7
0.001**	19.33	±1.966	CMC treated group	
0.000**	27.33	±1.966	Spirulina treated group	
	25.17	±2.23	Control group	
1.000	22.67	±2.25	Xanthan treated group	Day 12
1.000	22	±4.38	CMC treated group	
0.003 **	18.83	±2.64	Spirulina treated group	

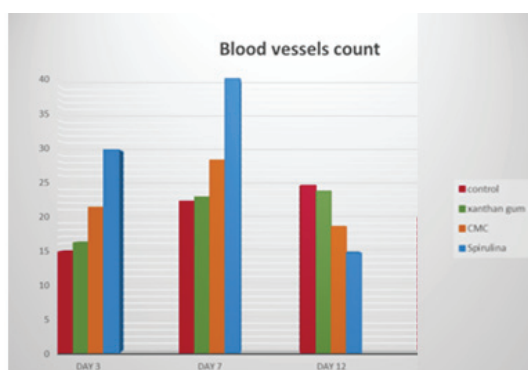
The number of fibroblasts is highly significant increased ($P \leq 0.001$) on day3 and 7 post-wounding and decreased on day 12 post-wounding compared to the control.

4- Area percent of collagen fibers deposition: Table (4) and in histogram (4).

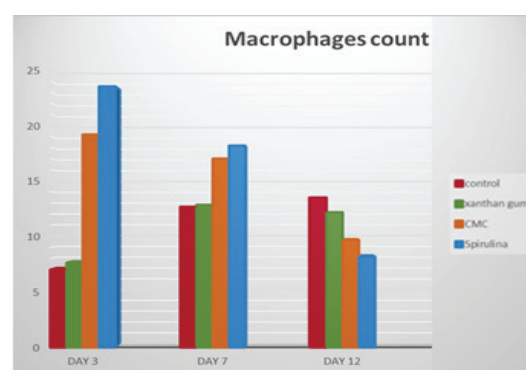
Table 4: Area % of collagen fibers deposition on day / high power field ((x100).

P value	Mean	±SD	Groups	Post-wounding days
	1.61	±0.48	Control group	
1.000	1.72	±0.78	Xanthan treated group	Day 3
1.000	2.95	±0.66	CMC treated group	
0.185	6.47	±1.37	Spirulina treated group	
	3.07	±0.65	Control group	
1.000	3.29	±0.53	Xanthan treated group	Day 7
0.164	7.98	±1.47	CMC treated group	
0.000**	16.13	±3.53	Spirulina treated group	
	18.74	±1.79	Control group	
1.000	18.75	±1.52	Xanthan treated group	Day 12
1.000	21.90	±1.76	CMC treated group	
0.003**	33.29	±4.01	Spirulina treated group	

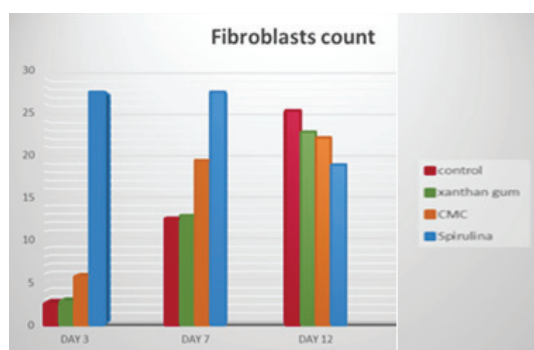
The area % of collagen fibers deposition is highly significant increased on day 7 and 12 post-wounding compared to control group.



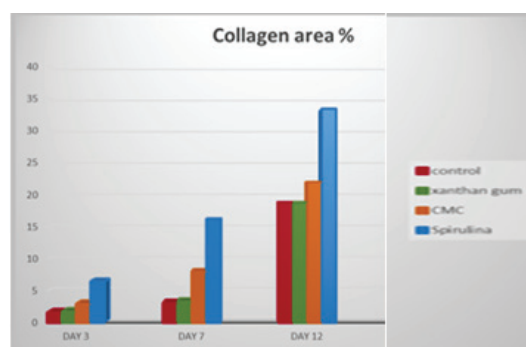
Histogram 1: Number of blood vessels / high power field.



Histogram 2: Number of macrophages / high power field.



Histogram 3: Number of fibroblasts/ high power field.



Histogram 4: Area % of collagen fibers deposition high power field.

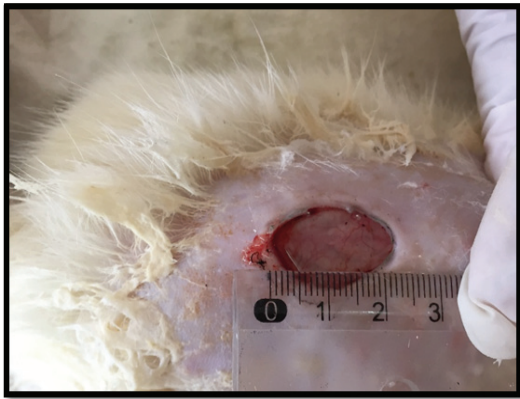


Fig. A: An excisional skin wound (2 cm in diameter) in the mid-back of the rats.

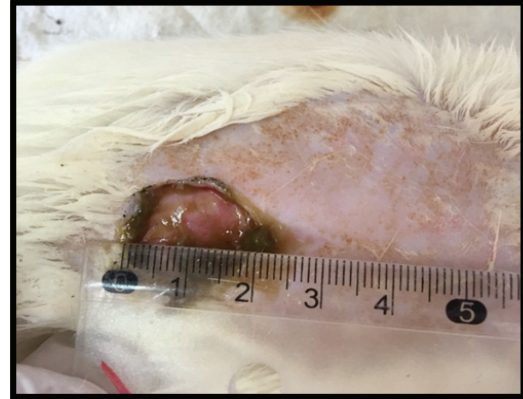


Fig. B: An excisional skin wound in rats treated with spirulina extract preparation.

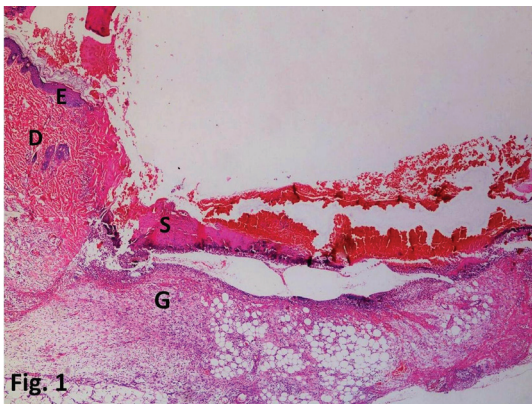


Fig. 1: A Photomicrograph of a section in rat's skin of group I (control) on day 3 post-wounding showing the site of the wound and adjacent nearby normal skin. The wound area is covered by thin scab (S) beneath which the granulation tissue (G) is observed. Note the epidermis (E) and the underlying dermis (D) of nearby skin. (Hx & E X40)

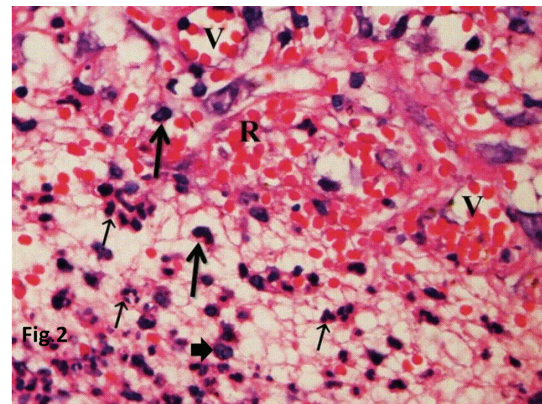


Fig. 2: A Photomicrograph of a part of section in rat's skin of group I on day 3 post-wounding showing that the granulation tissue contains many polymorphnuclear cells (thin arrows), macrophages (thick arrows), lymphocytes (arrow head) and blood vessels (V) with extravasated red blood cells (R). (Hx&EX1000)

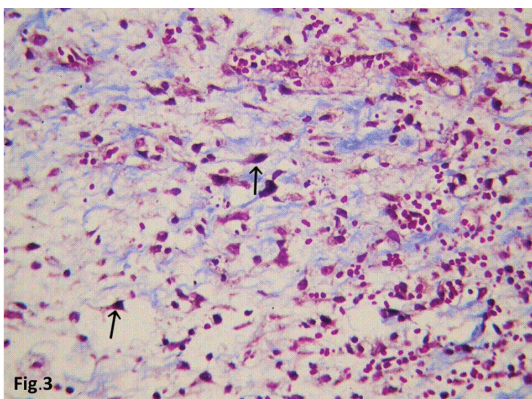


Fig. 3: A Photomicrograph of a part of section in rat's skin of group I on day 3 post-wounding showing few scattered collagen fibers at the site of the wound (blue coloration). Note the presence of fibroblasts (↑) and extravasation of red blood cells. (Mallory's trichrome X 400)

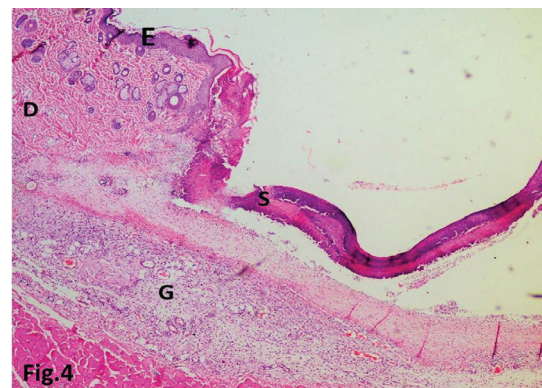


Fig. 4: A Photomicrograph of a section in rat's skin of group III (CMC treated group) on day 3 post-wounding showing the site of the wound and adjacent nearby normal skin. The wound area is covered by thin scab (S) that overlies the newly formed granulation tissue (G). The nearby skin shows the epidermis (E) and dermis (D). (Hx & E X40)

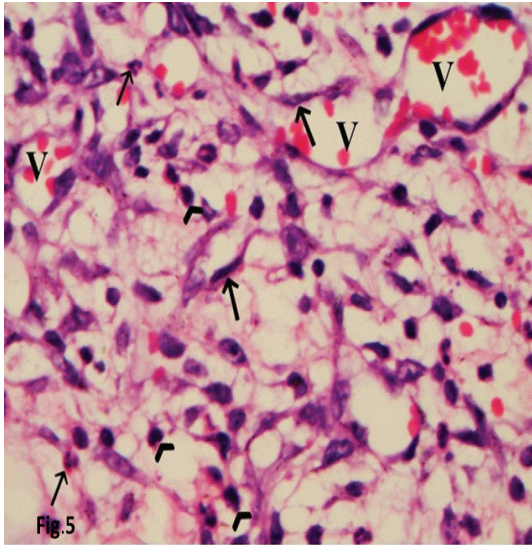


Fig. 5: A Photomicrograph of a part of section in rat's skin of group III on day 3 post-wounding, showing that the granulation tissue contains polymorphnuclear cells (thin arrows), many macrophages (arrow head), few fibroblasts (thick arrows)and multiple blood vessels (V). (Hx & E X1000)

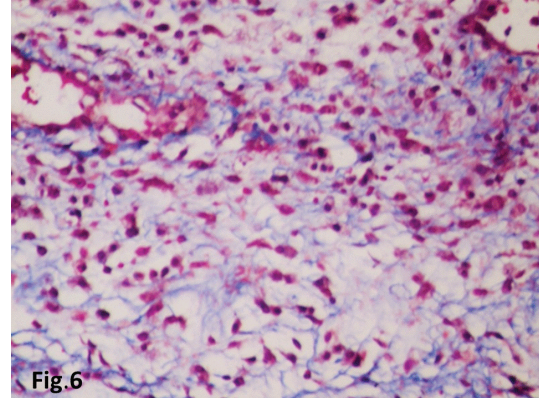


Fig. 6: A Photomicrograph of a section in rat's skin of group III on day 3 post-wounding showing few scattered collagen fibers (blue coloration) in the granulation tissue at the site of the wound. (Mallory's trichrome X 400)

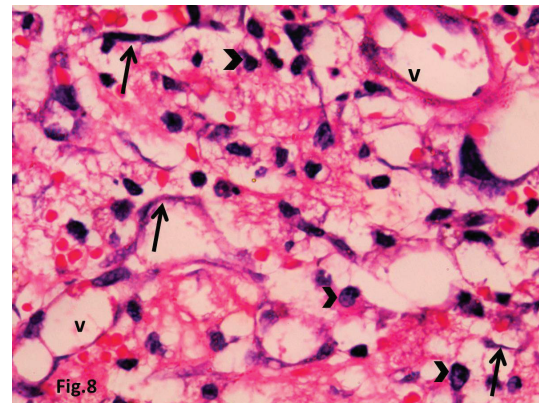


Fig. 8: A Photomicrograph of a section in rat's skin of group IV on day 3 post-wounding showing that the granulation tissue is formed of newly formed blood vessels (V), numerous macrophages (arrow head) and many fibroblasts (↑). (Hx&E X1000)

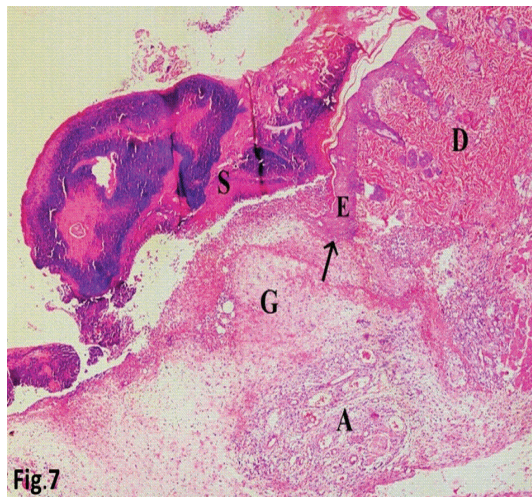


Fig. 7: A Photomicrograph of a section in rat's skin of group IV (spirulina treated group) on day 3 post-wounding showing the site of the wound and adjacent nearby normal skin. The wound area is covered by thick scab (S). The epidermis (E) at the cut edge is thickened and the beginning of re-epithelialization at the wound area can be seen (↑). The granulation tissue (G) is observed with an area of angiogenesis (A). The underlying dermis (D) of nearby skin is also noted. (Hx & E X40)

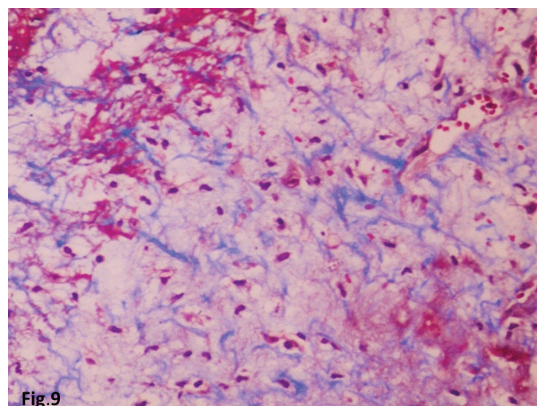


Fig. 9: A Photomicrograph of a section in rat's skin of group IV on day 3 post-wounding showing that the granulation tissue at the site of wound is formed of deeply stained irregularly distributed collagen fibers in the granulation tissue (blue coloration). (Mallory's trichrome X 400)

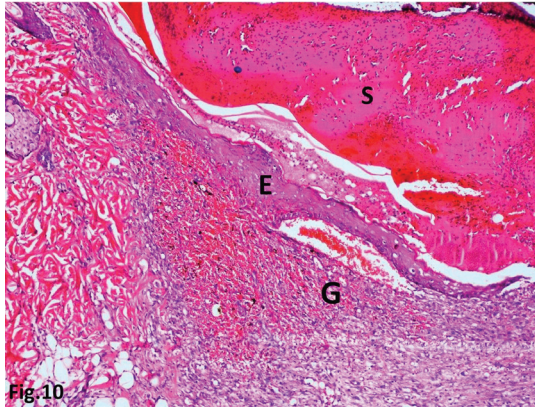


Fig. 10: A Photomicrograph of a section in rat's skin of group I (control group) on day 7 post-wounding, at the site of the wound showing re-epithelialization (E) beneath the scab (S). Note that, the newly formed epidermis shows poorly differentiated layers. The granulation tissue (G) exhibits numerous blood vessels. (Hx & E X100)

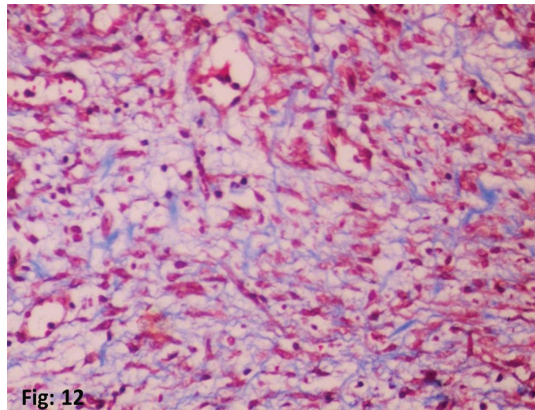


Fig. 12: A Photomicrograph of a part of section in rat's skin of group I on day 7 post-wounding, at the site of the wound showing few haphazardly scattered collagen fibers in the granulation tissue (blue coloration). (Mallory's trichrome X 400)

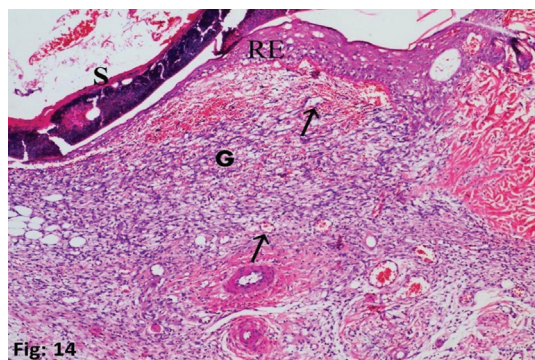


Fig. 14: A Photomicrograph of a section in rat's skin of group III on day 7 post-wounding showing poorly differentiated layers of newly formed epidermis (RE) beneath the scab (S). The arrows point to blood vessels (↑) in the granulation tissue (G). (Hx & E X100)

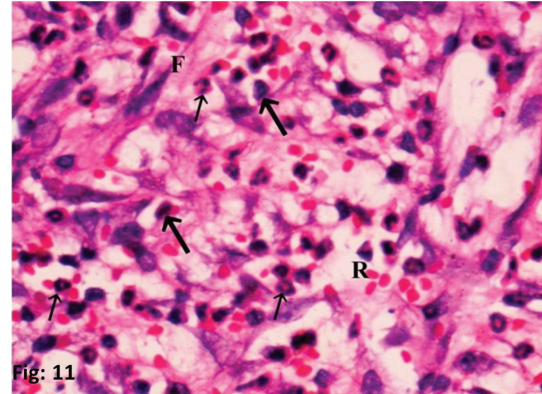


Fig. 11: A Photomicrograph of a section in rat's skin of group I on day 7 post-wounding showing that the granulation tissue contains many polymorphnuclear cells (thin arrows), macrophages (thick arrows), few fibroblasts (F) and extravasated red blood cells (R). (Hx & E X1000)

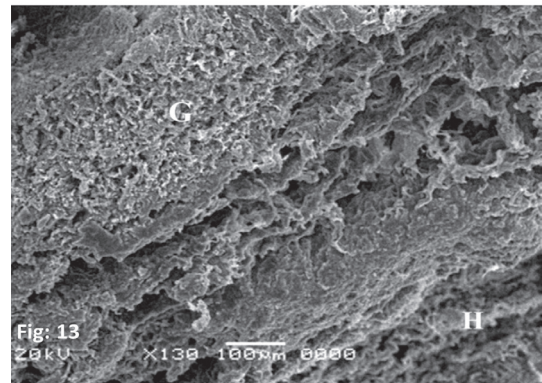


Fig. 13: A scanning electron-micrograph of a part of rat's skin of group I on day 7 post-wounding, showing the opened part of the wound without epithelialization. The underlying granulation tissue (G) contains few scattered collagen fibers separated by wide spaces. The hypodermis (H) is observed beneath the newly formed granulation tissue. (SEM X 130)

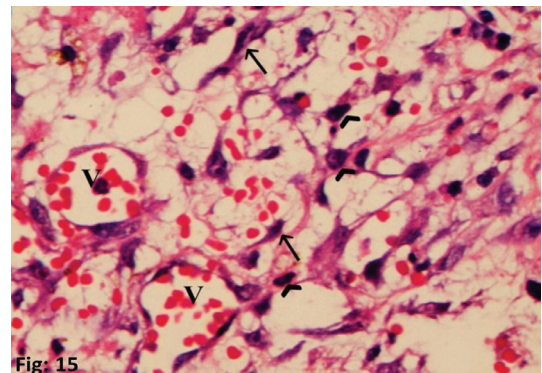


Fig. 15: A Photomicrograph of a section in rat's skin of group III on day 7 post-wounding showing that the granulation tissue contains numerous blood vessels (V), macrophages (arrow head) and fibroblasts (↑). (Hx & E X1000)

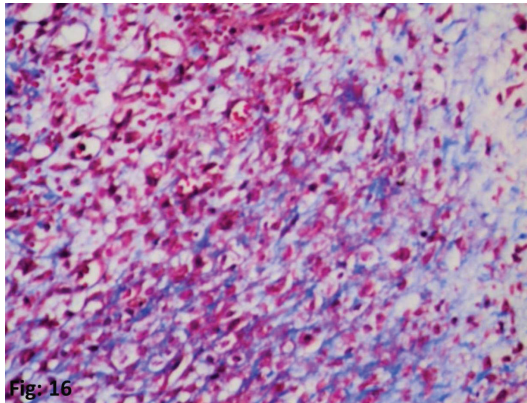


Fig. 16: A Photomicrograph of a section in rat's skin of group III on day 7 post-wounding, showing many scattered collagen fibers at the site of the wound (blue coloration). (Mallory's trichrome X 400)

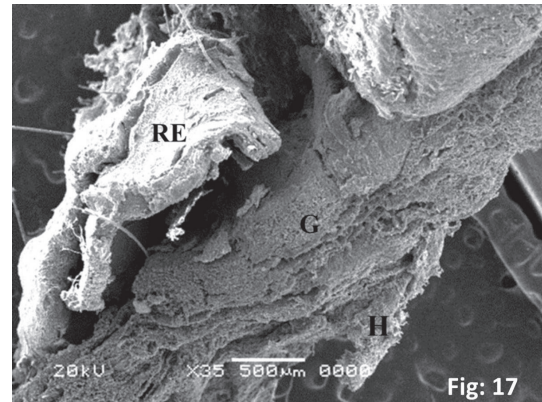


Fig. 17: A scanning electron-micrograph of part of rat's skin of group III on day 7 post-wounding showing the re-epithelialization (RE). The underlying granulation tissue (G) is formed of few wide spaced collagen fibers. The underlying hypodermis (H) is apparent. (SEM X 35)

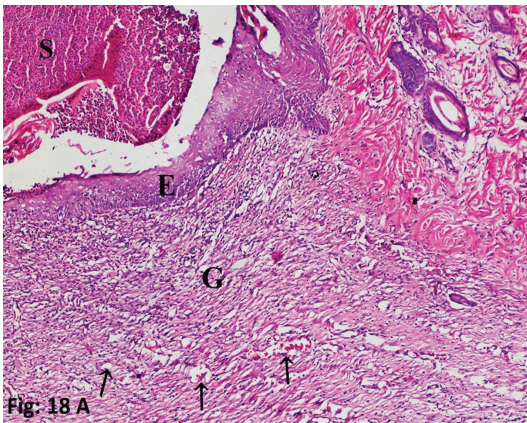


Fig. 18 :A, B: A Photomicrograph of a part of section in rat's skin of group IV on day 7 post-wounding at the site of the wound. (Fig.18A): showing the newly formed epidermis (E) beneath the scab (S) and the underlying granulation tissue (G) with numerous blood vessels (↑). (Fig.18B): showing regular arrangement of layers of newly formed epidermis; basal cell layer (B), prickle cell layer (P), granular cell layer (G) and the keratin on the top (K). Numerous blood vessels (V) and fibroblasts (↑) can be noticed in the granulation tissue. (Hx & E: A X100, B X400)

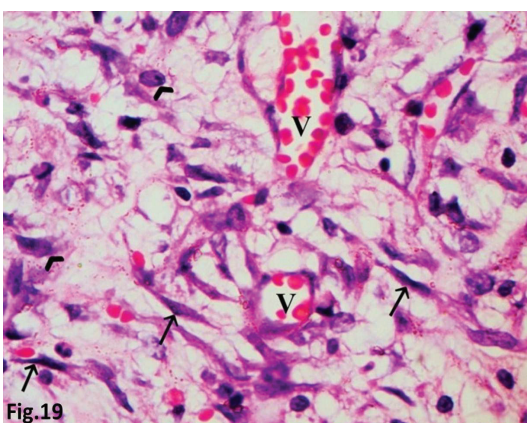
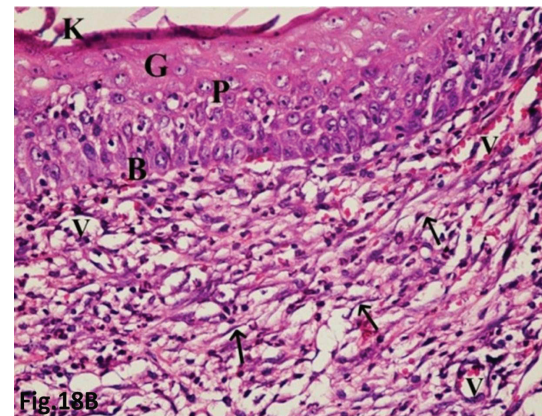


Fig. 19: A Photomicrograph of a part of section in rat's skin of group IV on day 7 post-wounding at the site of the wound, showing many blood vessels (V) and fibroblasts (↑) with few macrophages (arrow head) in the granulation tissue. (Hx & E X1000)

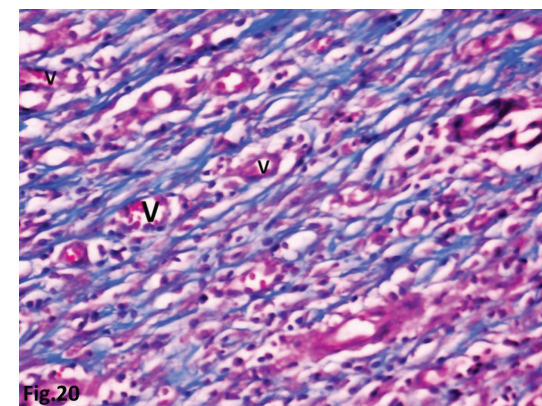


Fig. 20: A Photomicrograph of a section in rat's skin of group IV on day 7 post-wounding at the site of the wound, showing heavy deposition of collagen fibers (blue coloration). Note the presence of numerous blood vessels (V) in the granulation tissue. (Mallory's trichrome X 400)

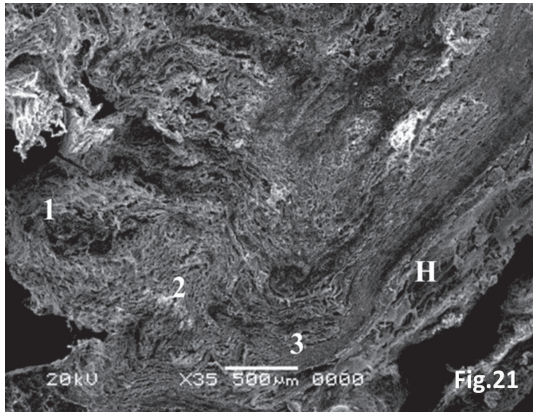


Fig. 21: A scanning electron-micrograph of part of rat's skin of group IV on day 7 post-wounding, showing that the granulation tissue is formed of three zones; sub-epithelial zone (1), middle zone (2) and deep zone (3). Note the underlying hypodermis (H). (SEM X 35)

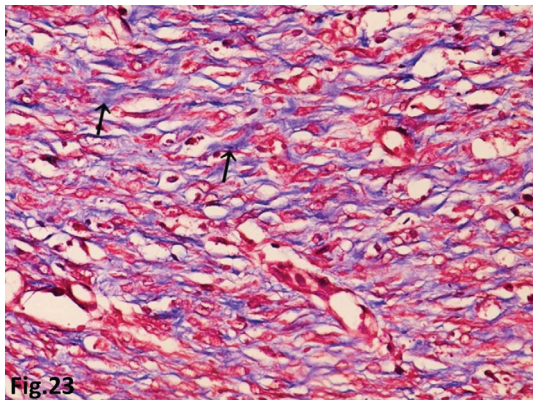


Fig. (23): A Photomicrograph of a section in rat's skin of group I on day 12 post-wounding, at the site of the wound, showing that the granulation tissue contains newly deposited collagen fibers (blue coloration). The collagen fibers were aligned horizontally (↑) in between the blood vessels. (Mallory's trichrome X 400)

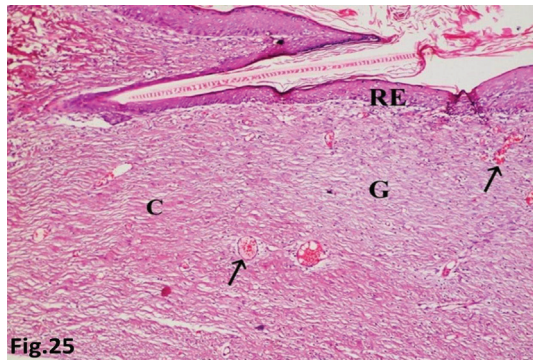


Fig. 25: A Photomicrograph of a section in rat's skin of group III (CMC treated group) on day 12 post-wounding, at the site of the wound showing that the re-epithelialization (RE) is not complete. The underlying granulation tissue (G) shows collagen fibers deposition (C) and numerous blood vessels (↑). (Hx & E X100)

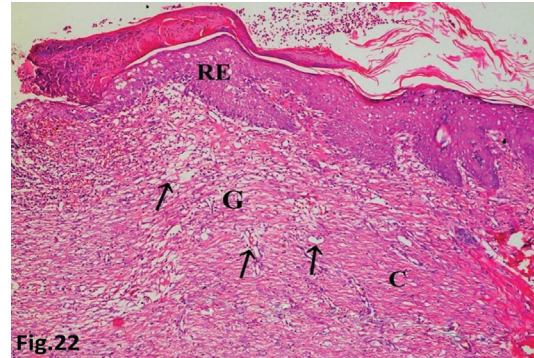


Fig. 22: A Photomicrograph of a section in rat's skin of group I (control group) on day 12 post-wounding, at the site of the wound showing complete absence of the scab. The granulation tissue (G) is covered by newly formed epidermis, but re-epithelialization (RE) is not complete. The granulation tissue (G) shows numerous blood vessels (↑) and collagen fibers deposition (C). (Hx & E X100)

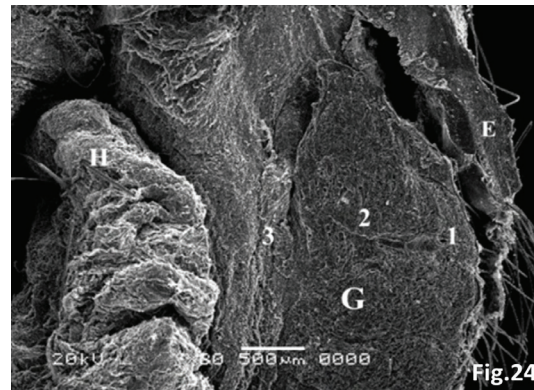


Fig. 24: A scanning electron-micrograph of a part in rat's skin of group I on day 12 post-wounding, showing that the granulation tissue (G) is discriminated into 3 zones, sub-epithelial zone (1), middle zone (2), deep zone (3). The underlying hypodermis (H) is also apparent. Note that the newly formed epidermis (E) is separated from the sub-epithelial zone. (SEM X 30)

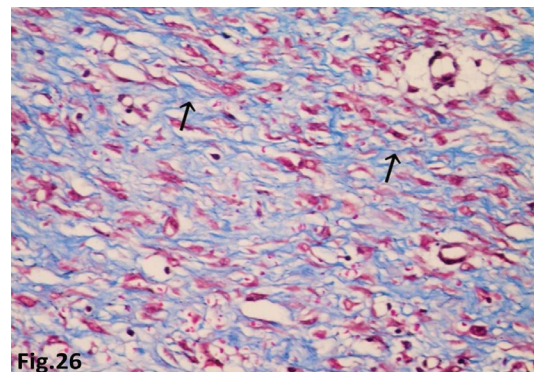


Fig. (26): A Photomicrograph of a section in rat's skin of group III on day 12 post-wounding, at the site of the wound, showing that the granulation tissue beneath the newly formed epidermis contains horizontally aligned, regularly arranged collagen fibers (↑). (Mallory's trichrome X 400)

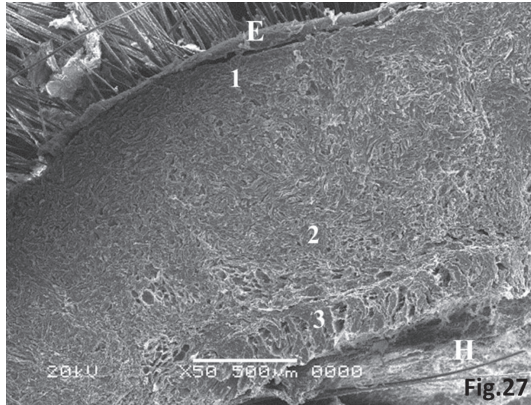


Fig. 27: A scanning electron-micrograph of rat's skin of group III on day 12 post-wounding, showing the newly formed epidermis (E) extending to cover the wound area. The underling granulation tissue is discriminated into 3 zones, sub-epithelial zone (1), middle zone (2), deep zone (3). The underling hypodermis (H) is also ap parent. (SEM X 50)



Fig. 28: A Photomicrograph of a section in rat's skin of group IV (spirulina treated group) on day 12 post-wounding, showing complete re-epithelialization (RE) of the narrow wound area. Note clear signs of increasing thickness of newly formed epidermis. The underlying regenerated dermis (D) shows few blood vessels (↑) . Note the dermis of nearby normal skin with sebaceous gland (arrow head). (Hx & E X100)

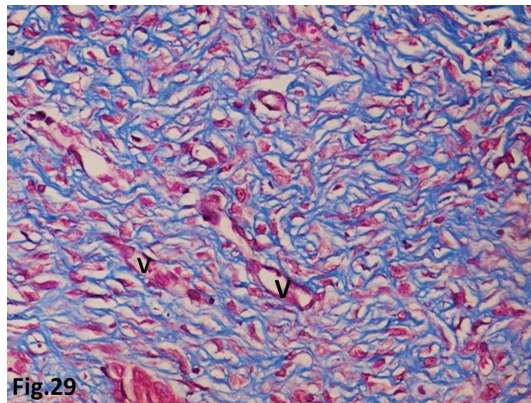


Fig. 29: A Photomicrograph of a section in rat's skin of group IV on day 12 post-wounding at the site of the wound, showing dense deposition of deeply stained regularly arranged collagen fibers(blue coloration) with intervening blood vessels (V) in the regenerated dermis. (Mallory's trichrome X 400)

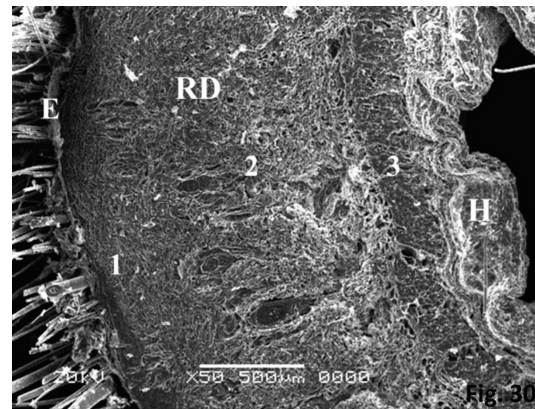


Fig. (30): A scanning electron-micrograph of part of rat's skin of group IV on day 12 post-wounding, showing the newly formed epidermis (E) covers the wound area. The underling regenerated dermis (RD) is discriminated into 3 clear zones, sub-epithelial zone (1), middle zone (2) and deep zone (3). The underling hypodermis (H) is also apparent. (SEM X50)

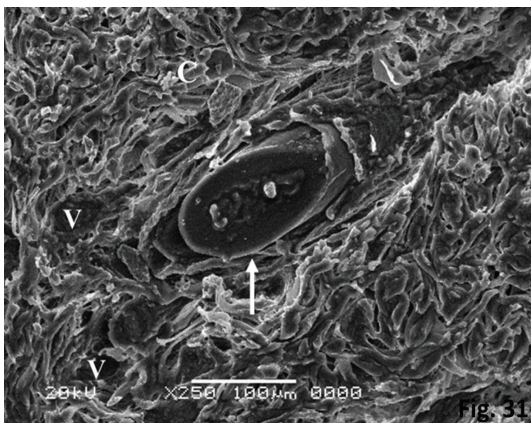


Fig. 31: A higher magnification of a part of Fig. (30), showing that the middle zone is formed of thick regular collagen fibers (C) deposited around the blood vessels (V). Note the appearance of newly formed hair follicle (↑).

DISCUSSION

The current work was designed to seek the role of spirulina extract on the healing process of excisional wound in adult male albino rats.

In the present work, wounds were examined in rats of spirulina treated groups versus CMC treated group and control group at three different time points (day 3, 7 and 12 post-wounding). Previous studies dealing with skin wound healing in rats were performed on similar post-wounding days by *Gur et al. (2013)* to evaluate the effect of topical application of spirulina extract .

The present study, re-epithelialization was observed on day 3 post-wounding in spirulina treated rats and on day 7 post-wounding in the control group and CMC treated rats. These results are in agreement with those of *Madhyastha et al. (2012)* and *Gur et al. (2013)* who noticed that the re-epithelialization started in rats at day 3 post-wounding in spirulina treated group and at day 7 post-wounding in the control group.

In the current work, the epidermis of spirulina treated rats regained its normal thickness and showed full differentiation on day 12 post-wounding with appearance of skin appendages (hair follicles). In the control and CMC treated groups, the granulation tissue is covered by newly formed epidermis, but re-epithelialization is not complete and the skin appendages failed to develop. These findings might be attributed to the role of spirulina extract in promotion of re-epithelialization and hair regeneration.

In hematoxylin and eosin stained sections in the present study, re-epithelialization was observed in the form of migrating keratinocytes from the basal layer of nearby thickened normal epidermis. These cells were noted across the wound gap deep to the scab and over the newly formed granulation tissue. In that account, *Braiman-Wisjsman et al. (2007)* declared that the migrating keratinocytes undergo subcellular modifications. These include disassembly of hemi-desmosomal links between epidermis and basement membrane, retraction of intracellular tonofilaments and keratin filaments, dissolution of most of desmosomes and formation of peripheral cytoplasmic actin filaments (lamellipodia).

Enoch et al. (2006) added that at the wound margin, keratinocytes begin to proliferate behind the actively migrating cells and the resulting dense hyperproliferative epithelium feeds the migrating sheets of keratinocytes. These events are regulated by three main molecular factors: growth factors, integrins and metalloproteases (MMPs).

Werner and Grose (2003) reported that growth factors released by fibroblasts ,macrophages and neutrophils appear to be very important for activating keratinocytes at the wound margins. These include platelet-derived growth factor (PDGF), transforming growth factor beta (TGF- β) and epidermal growth factor (EGF). Moreover, expression and activation of MMPs promotes degradation and modification of extracellular matrix proteins at the wound site, facilitating cell migration. Then, BM proteins reappear in a zipper-like fashion, from the margin of the wound inward and epidermal cells firmly attached to the reestablished BM and underlying dermis (*Song et al., 2010*).

In this study the granulation tissue in the wound area on day 3 post-wounding included cellular elements (PMNL, macrophages and fibroblasts), new small blood vessels (angiogenesis) and collagen fibers in rats of all examined groups.

In that respect, *Park and Barbul (2004)* described similar components of the granulation tissue during wound healing that were embedded in loose extra cellular matrix of collagen, fibronectin and hyaluronic acid. The authors added that the granulation tissue appeared with a classic redness due to presence of angiogenesis and might be traumatized easily.

In the present work, few PMNL were observed in the granulation tissue in the wound area of spirulina treated group on day 3 post-wounding and rarely encountered on day 7 post-wounding.

Early disappearance of inflammation in spirulina treated wounds might facilitate earlier progress to the next phase of wound healing as reduced inflammation promotes regeneration rather than scarring. This concept coincided with the findings of *Edefia et al. (2015)* who confirmed

the anti-inflammatory effect of spirulina extract versus several herbal extracts in day 7 post-wounding. The authors attributed this anti-inflammatory effect to the presence of phycocyanin (PC) in the spirulina extract. This PC has received much attention as a potent inhibitor of COX-2 as reported by *Romay et al. (2003)*. COX-2 is an inducible form that participates in inflammation, and is responsible for the infiltration of neutrophils and for the production of prostaglandins, Nitric Oxide. (NO) and tumor necrosis factor alpha (TNF- α) at inflammation sites as stated by *Shih et al. (2009)*. In the present work, heavy infiltrates of PMNL (neutrophils) were observed on day 3 post-wounding in the control group and CMC treated group especially in the superficial layer of the granulation tissue just beneath the scab and few cells were still encountered on day 12 post-wounding. This finding denotes persistence of inflammatory phase in the control group and CMC treated group.

In that respect, *Park and Barbul (2004)* declared that in non-infected wounds the number of neutrophils decrease rapidly after the third day and undergo apoptosis. Then these cells are phagocytosed by macrophages.

In the present work, the number of macrophages in spirulina treated wounds showed a highly significant increase on day 3 post-wounding and highly significant decrease on day 12 post-wounding compared to the control group. These findings are in line with those of *Toyokawa et al. (2003)* who evaluated the effect of far-infrared ray (FIR) on the wound healing of the rats on day 3 and day 12 post-wounding.

In contrary, *De Masi et al. (2016)* reported no evident increase in the number of macrophages in rat's wound injected by growth factors on day 3 post-wounding, despite the great importance of its presence in that day. *Park and Barbul (2004)* declared that early in the inflammatory phase macrophages play an important role in promoting wound healing by clearing the apoptotic cells (neutrophils) and releasing cytokines e.g. TNF- α and IL-6 against pathogens. In addition macrophages stimulate keratinocytes, fibroblasts and angiogenesis to promote tissue regeneration in proliferative phase.

In the current study, there was a statistical increase on day 3 and day 7 post-wounding on the number of newly formed blood vessels which was highly significant in spirulina treated wounds and non-significant in CMC treated wounds compared to the control group. These findings are coincided with those of *Panigrahi et al. (2011)* who reported that the capillary density in spirulina treated wounds was significantly higher than in the untreated wounds in the first 7 days post-wounding. This implies a potential significant advantage for spirulina extract to stimulate endothelial cells and angiogenesis in tissue repair.

Angiogenesis is very important as it improves circulation to the wound site thus providing oxygen and nutrients essential for the healing process, as explained by *Malinda et al. (2008)*.

In the present work, the number of the new blood vessels showed a statistical decrease on day 12 post-wounding which was highly significant in spirulina treated wounds and non-significant in CMC treated wounds compared to the control group.

This finding could be explained by *Bao et al. (2009)* who reported that once the wound is granulated, angiogenesis ceases and blood vessels decline as endothelial cells undergo apoptosis.

In the present study, there was a statistical increase in the number of fibroblasts which was highly significant in spirulina treated wounds and non-significant in CMC treated wounds on day 3 post-wounding compared to the control group. However, on day 7 post-wounding this increase was highly significant on both groups compared to the control group. Similarly, *De Masi et al. (2016)* noted significant increase in the number of fibroblasts in the rat's wound injected by growth factors on day 3 and day 7 post-wounding compared to the control group.

The main function of fibroblasts is to produce collagen reported by *Diegelmann and Evans (2004)*. In the current study, there was a statistical decrease of the number of fibroblasts on day 12 post-wounding which was highly significant in

spirulina treated wounds and non-significant in CMC treated wounds compared to the control group. Accordingly, *De Masi et al. (2016)* detected a significant decrease in the number of fibroblasts in the rat's wounds injected by growth factors on day 15 post-wounding compared to the control group.

These findings could be explained by *Barrientos et al. (2008)* who noted that when collagen density in the wound reaches a certain threshold, fibroblast proliferation and collagen synthesis are suppressed and the remodeling process begins. *Gabbiani (2003)* stated that the fibroblasts differentiate during normal wound healing into myofibroblasts which are transiently present at the wound site causing wound contraction and restoration of tissue integrity.

In that respect, *An et al. (2015)* declared the importance of phycocyanin which inhibits the persistence of myofibroblast in wound healing by controlling the inflammatory signal to macrophage that produces TGF- β . The authors suggested the potential application of phycocyanin for anti-fibrosis therapy and scar modulating agent.

In the current study, the area % of collagen fibers deposition in Mallory's trichrome stained sections, revealed a statistical increase which was non-significant on day 3 post-wounding and highly significant on day 7 and 12 post-wounding in spirulina treated wounds compared to control group. These findings are coincided with those of *Gur et al. (2013)*.

In the current study, SEM of spirulina treated wounds showed early discrimination of the collagen fibers into three zones on day 7 post-wounding. These zones were arranged as sub-epithelial zone, middle zone, and deep zone. On day 12 post-wounding, these three zones become more prominent in spirulina treated group. These findings are explained by *Meyer et al. (1982)* who use SEM and reported the presence of three structural zones in the collagen fibers within the dermis of the domestic pigs. These zones are: superficial zone – a thin layer of fine fibers adjacent to the epidermis, mid zone – a thicker layer of densely packed coarse fibers and a deep zone – a loosely arranged layer of coarse fibers. This newly formed superficial fine fibrous network was found to be the same kind

as seen in normal papillary dermis. While those coarse fibers of the mid and deep zones tended to assemble into thick, curly bundles forming rough and coarse network and were found to be like that of reticular dermis.

In the present study, SEM on day 12 post wounding revealed that the sub-epithelial zone of collagen fibers was adherent to the newly formed epidermis in spirulina treated group and detached in CMC treated group and control group. These findings are explained by SEM performed by *Yamamoto et al. (2004)* who revealed that the two dermal layers (papillary and reticular) follow different developmental processes during wound healing. Despite the papillary dermis being a dermal component, its neoformation proceeds in conjunction with epithelialization. The papillary dermis and the epidermis were found to be related during the wound healing process. The authors called them, including the basement membrane, the epithelialization unit. The healing process was completed with total coverage of the wound by this unit.

Reinke and Sorg (2012) stated that collagenases and proteases cleave and degrade collagen fibers type III. These fibers are gradually absorbed and replaced with type I collagen fibers, increasing the strength, organization and thickness of the extracellular matrix.

The beneficial effects of spirulina extract on wound healing in the current study may be due to its immune modulating ability and antioxidant activity that results in reduced inflammation, neutrophil recruitment, and consequently, reduction in oxidative stress providing a favorable environment for tissue healing as reported by *Bermejo-Bescós et al. (2008)*. The authors added that the phycocyanin is the main component responsible for the antioxidant activity of the spirulina platensis extract which can effectively eliminate hydroxyl and oxygen free radicals.

Similarly, *Madhyastha et al. (2012)* showed that spirulina extract inhibited the production of reactive oxygen species, thus promotes wound healing. This antioxidative activity of spirulina extract was beneficial for wound healing and was also demonstrated by other studies (*Li et al., 2005, El-Baky et al., 2009; Palaza et al., 2009*).

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تأثير مستخلص سبيرولينا على التئام الجروح الجلدية في الجرذان البيضاء البالغة: دراسة بالمجهر الضوئي والمجهر الإلكتروني الماسح

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ملخص البحث

تعتبر الجروح من المشاكل الطبية الشائعة. ويؤدي الإلتئام البطيء للجروح الى مشاكل طبية واقتصادية. تعد طحالب سبيرولينا أحد المكملات الغذائية الهامة و احد مضادات الاكسدة الفعالة و تستخدم في علاج بعض الامراض. لذلك كان الهدف من البحث هو دراسة تأثير الإستخدام الموضعي لمستخلص سبيرولينا في التئام الجروح الجلدية المستئصلة في نموذج الجرذان. في هذه الدراسة تم عمل جرح الجلد المستئصل وقطره 2 سم على منتصف الظهر في 72 من ذكور الجرذان. تم تقسيم الحيوانات إلى أربعة مجموعات (18 جرذا لكل مجموعة): المجموعة الأولى (المجموعة الضابطة)، المجموعة الثانية (المجموعة المعالجة بصمغ زنتان) الذي كان يستخدم كمذيب للسبيرولينا، المجموعة الثالثة (المجموعة المعالجة بمادة كاربوكسي ميثيل سليولوز) والتي كانت تمثل المجموعة المرجعية و المجموعة الرابعة (المجموعة المعالجة بسبيرولينا). تم علاج الحيوانات من المجموعة الثانية و الثالثة و الرابعة باستخدام 0.2 ملييلتر من كل مادة. في هذه المجموعات تم إستخدام المواد موضعيا مرتين يوميا حتى نهاية كل فترة تجريبية. تم تقسيم كل مجموعة الى ثلاثة مجموعات فرعية والتي تمثل أيام النحر 3 و 7 و 12 يوما على التوالي. بعد التضحية بالحيوانات، تم جمع عينات من موقع الجرح و 2 ملييمتر من الجلد الطبيعي المجاور. تم تمرير بعض منهم في الفورمالين المتعادل 10 ٪ لعمل قطاعات البارافين التي تم صبغتها بالهيماتوكسيلين و الايوسين و ثلاثي الألوان مالوري، في حين تم تمرير العينات الأخرى في 2.5 ٪ جلوتارالدهيد وتجهيزها للفحص بالمجهر الإلكتروني الماسح. وقد أجريت الدراسة المورفومترية باستخدام محلل الصورة لقياس عدد كل من: الأوعية الدموية التي تكونت حديثا والخلايا الملتهمة والخلايا الليفية وأيضا لحساب النسبة المئوية من مساحة ترسب ألياف الكولاجين. وقد تم التحليل الإحصائي باستخدام اختبار أنوفا. أظهرت المقاطع التي تمت صبغتها بالهيماتوكسيلين و الايوسين في اليوم الثالث بعد إحداث الجرح بداية إعادة تشكيل النسيج الظهاري في المجموعة التي تلقت العلاج بسبيرولينا بينما لوحظ غياب البشرة في منطقة الجرح في جميع المجموعات. و في اليوم السابع لوحظ بدايه تكوين الطبقة الظهارية في المجموعة الضابطة الأولى و المجموعة الثالثة بتباين غير ناضج بينما امتدت هذه الطبقة في المجموعة المعالجة بسبيرولينا الي مركز الجرح وأظهرت تبايناً ناضجاً. هذا وقد أظهرت المجموعة المعالجة بسبيرولينا في اليوم الثاني عشر بعد إحداث الجرح أن منطقه الجرح تم تغطيتها كاملا بالطبقة الظهارية السميكة مع ظهور زوائد الجلد (بصيلات الشعر) والذي لم يلاحظ في باقي المجموعات. وأظهر تحليل الصور زيادة في عدد الأوعية الدموية التي تكونت حديثا وفي عدد من الخلايا الليفية والخلايا الملتهمة في اليوم الثالث و اليوم السابع من إحداث الجرح وكانت هذه الزيادة ذات دلالة إحصائية في المجموعة المعالجة بسبيرولينا مقارنة مع المجموعة المعالجة بمادة كاربوكسي ميثيل سليولوز و المجموعة الضابطة. من ناحية أخرى، أظهرت هذه القياسات تناقص في اليوم الثاني عشر من إحداث الجرح وكان ذا دلالة إحصائية في الجروح المعالجة بسبيرولينا مقارنة مع باقي المجموعات. بفحص المقاطع المصبوغة بثلاثي الألوان مالوري في المجموعة المعالجة بسبيرولينا كان ترسب ألياف الكولاجين متناثرة في اليوم الثالث و منتظماً ومرتباً أفقياً في إتجاه مواز لسطح الجرح في اليوم السابع و الثاني عشر بخلاف باقي المجموعات و وجدت زيادة ليست ذات دلالة إحصائية في النسبة المئوية لترسب ألياف الكولاجين في اليوم الثالث في كلٍ من المجموعة المعالجة بمادة كاربوكسي ميثيل سليولوز و المجموعة المعالجة بسبيرولينا مقارنة بالمجموعة الضابطة. اما في اليوم السابع والثاني عشر وجدت زيادة ذات دلالة إحصائية عالية في المجموعة المعالجة بسبيرولينا مقارنة بباقي المجموعات. كما ظهرت ألياف الكولاجين متمارزه في ثلاث طبقات بالفحص بالميكروسكوب الإلكتروني الماسح في اليوم السابع و الثاني عشر في المجموعة المعالجة بسبيرولينا مقارنة مع باقي المجموعات.