Journal of Positive School Psychology
2022, Vol. 6, No. 5, 9841-9852
http://journalppw.com

Comparison of the effect of *Aloe vera* nanofibers enriched samples with *Arnebia euchroma* extracts with other treatment and a quantitative study of collagen I and collagen III genes expression in the skin of mice after micro-needling

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Abstract

Background: Mechanical injuries and aging, causes changes in skin collagen. In the experimental and traditional methods, herbs and aloe vera gel are used to repair and rejuvenation of skin.

Objective: In this study, the effect of and aloe vera nanofibers and their enriched nanofibers with extract (*Arnebia euchorma*) and other treatments were investigated and compared on the expression of collagen I and collagen III genes in the skin mice after micro needling.

Methods: The experiments were performed under standard conditions. Micro needling was performed on each tested mouse and each was treated for 48 hours. expression of collagen I and collagen III genes was evaluated by RT- PCR method. According to the obtained results, with treatments, Extract, gels, nanofibers and enriched nanofibers has a significant increase compared to the model group.

Resules: Micro needling stimulated the skin and it allows to penetrate the skin by creating very small pores. These materials were also gradually released from nanofibers. Shikonin probebly inhibited the pathway NF-κB. Glucomannan, increases collagen I and collagen III genes expression at the injury site by affecting the MAP ERK pathway.

Conclusion: The process of expressing collagen I and collagen III gene is essential in repairing and rejuvenating the skin.

Keywords: Microneedling, Nanofibers, *Aloe vera*, *Arnebia euchroma*, Collagene

INTRODUCTION

Preserving the natural structure of the skin, especially the facial skin, is of great importance in social relationships. Cosmetic treatments and plastic surgeries have become a huge business all over the world to refresh or remove skin lesions. The extracellular part of animal tissues is called the Extra Cellular Matrix (ECM). Wound healing, fibrosis repair, proliferation, migration, cell differentiation, and morphogenesis are the applications of ECM. ECM is also considered as storage of growth factors [1]. Collagen accounts for about 30% of body weight. This protein plays an important role in wound healing, cell proliferation, migration, and cell differentiation. Collagen gives the ECM

the power to expand and elastin gives it elasticity, allowing it to stretch and then return to its original position. Collagen type I (80%) and collagen III are the most common type of collagen in the skin matrix.

Microneedling is one of the rejuvenating methods to stimulate the skin to produce collagen. In this technique, the fine needles of a microneedling device create small pores to a certain depth of the skin. So, there is the possibility of mesotherapy and drug delivery or investigating the effects of environmental factors such as growth factors through very small pores created [2, 3].

The leaves of Aloe vera (*Aloe barbadensis Miller*) contain mucilage tissue. This mucilage possesses some glycoproteins that accelerate wound healing and prevent swelling and pain. Its restorative properties are attributed to a compound called glucomannan, which is rich in mannose [4]. In one study, aloe vera gelatin nanofibers were synthesized and used for wound healing. These nanofibers inhibited aerosol particles using the trapping mechanism, provided a suitable pattern for vapor transfer, and prevented bacteria from penetrating the wound surface. These fibers are important in drug delivery because of their high surface area [5].

Arnebia euchroma is rich in Naphthoquinone, shikonin, and alkaline [2]. These substances and their derivatives have biological properties such as wound healing, antimicrobial and anti-inflammatory effects [6], antifungal [7] and antiviral activities (such as influenza and AIDS) [4, 8], and anti-cancer properties [9].

When aloe vera gel is applied to the skin, the active ingredients are unlikely to penetrate the healthy epidermis. Also, as the gel dries, the release of glucomannan decreases. In microneedling, the skin tissue is stimulated by a minor injury, and the pores created can be used for targeted medication. Therefore, in this study, by combining the mentioned materials and methods, microneedling was performed on rat skin and by applying aloe vera gelatin nanofibers enriched with *Arnebia euchroma* hexane extract on the skin, effective drug delivery to the target tissue was investigated.

The quantitative tissue RNA index of the treatment and control groups was used to evaluate the collagen, as well as studying and evaluating the gene expression.

This study aimed to compare the traditional use of aloe vera with the mentioned methods for expressing the collagen I gene in the skin of laboratory male mice. Gene expression in the control group with healthy skin has a natural quantity and quality. However, after microneedling, the skin of the treatment groups was destroyed and the expression of the collagen I gene was minimized in them. In this study, treatment groups were compared to investigate the role of nanofiber dressings, enriched nanofibers, and aloe vera gel on the modulation and expression of the Type I collagen gene in skin repair and rejuvenation after microneedling.

Materials and Methods

Animal

In this experimental study, 30 male Balb/c mice weighing 22 ± 2 g were used. The animals were divided into five independent groups each consisting of six animals as follows:

1- Model group: Microneedling + saline treatment, 2- Extract group: Microneedling + *Arnebia euchroma* root hexane extract (5%) treatment, 3- Microneedling + aloe vera gel treatment 4- Microneedling + aloe vera nanofibers treatment, 5- Microneedling + aloe vera nanofibers enriched + *Arnebia euchroma* root hexane extract (5%) treatment.

This study was performed on license and ethical identity IR.IAU.TNB.REC.1400.116. The animals were kept in laboratory conditions, under 12-h/12-h light-dark cycle conditions at a temperature of 22 ± 2°C, a humidity of 50%, and with ad libitum water and food. The model and treatment groups were anesthetized using an intraperitoneal injection of ketamine and Xylazine. Their skin was microneedled after shaving their back hair. Treatment was performed every 24 h for 48 h.

Synthesis of nanofibers

Gelatin and aloe vera gel powder were used to synthesize aloe vera gelatin nanofibers. First, a 10 v/v% solution of bovine gelatin with 90% acetic acid was prepared. Then, a 5 wt.% solution of aloe vera powder gel with 90% acetic acid was prepared. A third solution was obtained by mixing an equal volume of the solutions made. The solution was sucked into a 5 ml syringe and loaded into an electrospinning device. Nanofibers

were collected on aluminum foil with a collection rate of 0.5 ml/sec, a rotation of 100 rpm, and a voltage of 15 kv over 4 h.

Sampling

For molecular studies, sampling was done using a 3-mm punch after animals were anesthetized.

Total mRNA extraction

To evaluate gene expression, RNA was first extracted from the tissues of each animal according to the protocol of the manufacturer (Kiagene, Germany). About 200-300 µl of Kiazol was added to the target tissue and kept at -80℃ for 24 h. The pellet in the cryotube was then crushed by a sampler tip and then pipetted. About 100 µl of chloroform was then added to cleave the cells. After 1 min, the solution was centrifuged at 12,000 g for 10 min and three layers were observed. The transparent section at the top of the tube contained RNA, the white section in the middle of the tube contained the decomposed tissue, and the pink section at the bottom of the tube contained the Kiazol.

The transparent section was gently removed and placed in a microtube. About 1 ml of isopropanol was then poured onto RNA and stirred for 1 min. Isopropanol and RNA are transparent, but they form a turbid liquid after mixing. After adding isopropanol, the samples were centrifuged at 12,000 g for 10 min. The supernatant was removed and 1 ml of 70% alcohol was added to the mix. After mixing the solution in a closed tube, it was centrifuged at 75 g for 10 min. The supernatant was then removed using a sampler and the resulting pellet was dried in a microtube. To dissolve the RNA, 20 µl of 60℃-distilled water was poured onto the pellet. It was then pipetted slightly using a sampler and placed on a plate with a temperature of 60℃ for 5 min. The extracted RNA was stored at -80 ℃ until use.

Evaluation of purity of RNA extracted using spectrophotometry

In this method, purity of RNA sample was investigated using the property of light absorption at a wavelength of 260 nm and the following formula: C (µg/µl) = A260 ×ε×d/1000. The amount of impurities due to the presence of protein or DNA in the RNA solution is calculated using the ratio of A260 to A280. In a pure RNA sample, this ratio is equal to 2±0.15 while in a pure DNA sample is equal to 1.8 ± 0.15. If the calculated ratio is less than the standard value, the sample is contaminated by protein.

cDNA synthesis: The prepared structure was exposed to reverse transcriptase enzyme activity. The reverse transcriptase enzyme synthesizes cDNA by scanning the mRNA strand and placing the deoxyribonucleic virus in front of each mRNA nucleotide.

Preparation

About 2 µg of RNA template and 1 µl of primer were added to 12 µl of DEPC water. The resulting solution was kept at 65 ℃ for 5 min and then placed on ice. Then, 4 µl of reaction buffer, 1 µl of ribonuclease, 2 µl of dNTP, and 1 µl of reverse transcriptase enzyme were added and incubated at 25℃ for 5 min. Next, it was then kept at 42°C for 60 min. After 5 min at 70°C, the reaction was stopped and the product was kept at -20°C.

Primers designing

In the present research, specific primers with the following specifications were used to evaluate the expression of the type II collagen gene and compare it with the GAPDH internal control gene. OLIGO 7 software was used to design the primer. To confirm the specific function of genes, we used BLAST at the NCBI site. The collagen I access code is NM-007742.4 and the resulting fragment length is 151 bp.

Col I-f	GGACTTGTGTGAATTGTTGGGG
Col I -r	GTGGAGAGAGAGTAGAGAGTGG

The collagen III access code is XM-021152688.2 and the resulting fragment length is 195 bp.

Col III-f	GGTGCTAAAGGAGAAAGAGGTG
Col III-r	ATTGACAGGAGGACCAGGAAGA

GAPDH gene was also used as an internal control gene. The accession code is NM-001289726.1 and the length of the resulting fragment is 124 bp.

Gapdh-f	CCCTTAAGAGGGATGCTGCC
Gapdh-r	TACGGCCAAATCCGTTCACA

Polymerase Chain Reaction (PCR)

In a polymerase chain reaction, DNA molecules are amplified in laboratory conditions using enzymes. This method involves repetitive cycles that are performed using a series of primers and DNA polymerase. Therefore, the DNA polymerization reaction of the template is performed. To produce DNA fragments, the cycles are performed to a point where detection is possible.

Real-Time PCR

Each PCR reaction is performed using Step One PCR Master Mix. Moreover, SYBER Green (Applied Biosystems, Sequences Detection Systems, Foster City CA) was performed according to the manufacturer's protocol. In this respect, 40 cycles were considered for each Real-Time PCR. The temperature of each cycle was set to 94℃ for 20 s, 60 ℃ for 30 s, and 72℃ for 30 s. The melt curve of PCR reactions was evaluated to investigate the contaminants in each reaction. In this study, a specific primer of Type I collagen was used. The expression ratio of the studied gene and the internal control gene was evaluated by the comparative threshold cycle (CT) method. Fold change was calculated using the following relations [10, 11].

$$\Delta\Delta CT = (CT_{target}\text{-}CT_{reference})_{Time\ X}\text{-}(CT_{target}\text{-}CT_{reference})_{Time} \quad (1)$$

$$R = 2^{-(\Delta\Delta CT)} \quad (2)$$

To calculate the significance level of gene expression differences between groups, ANOVA and Tukey tests were used. GenEx6 software was used to evaluate gene expression differences. Statistical analysis was performed using SPSS 21 software. Eventually, GraphPad Prism 8 software was used to draw the graphs.

Results

The synthesized nanofibers were characterized using scanning electron microscopy (SEM). As presented in Figure 1, the size of the synthesized aloe vera gelatin nanofibers was between 371 and 479 nm. SEM images show the regular three-dimensional structure of aloe vera gelatin nanofibers. It was also found that the appearance of aloe vera gelatin nanofiber filaments was perfectly arranged. The presence of holes in SEM electron microscope images suggests the self-arrangement of the filaments (Figure 1).

FTIR: (Fourier transform infrared spectroscopy)

FTIR test was performed to evaluate the functional groups. The results of the FTIR test indicated no change in the functional groups of gelatin-aloe vera nanofibers. In this way, all properties of gelatin and aloe vera were preserved in the nanofibers.

FTIR spectra of aloe vera powder, gelatin nanofibers, and gelatin-aloe-vera nanofibers were studied to investigate the functional groups of gelatin and aloe vera and their interaction in gelatin-aloe vera (Figure 2). Gelatin protein-polymer has amide groups, with their index peaks appearing at the wavenumber of 3350 cm^{-1} (-NH stretching of amide I and II), 1652 cm^{-1} (C = O stretching and -NH bending of amide I). Also, their strong peaks appear at 1537 cm^{-1} (a combination of -NH bending and C – N stretching of amide II) and 1451 cm^{-1} (a combination of aliphatic and COO-groups). On the other hand, the FTIR spectrum of aloe vera has sharp peaks in wave numbers 3382 cm^{-1} (-NH amine group), 2926 cm^{-1} (aliphatic group), 1723 cm^{-1} (C = O related to O-acetyl ester), 1600 cm^{-1} (C = C related to the aloin vinyl group), and 1097 cm^{-1} and 1254 cm^{-1} (C-O-C of glucoside units and the ester and phenol groups). The absence of a new peak in the gelatin-aloe vera nanofiber spectrum indicates the lack of any chemical reaction between the two components (Figure 2).

In Figure 3, the model group was used as a reference for calculating fold change. According to the Figure 3, the expression level of the Type I collagen gene in all treatment groups is more than compared to the model group. It indicates a

significant increase in Type I collagen gene expression in the treatment groups compared to the Model group (reference). To evaluate and analyze the differences in gene expression between groups, an ANOVA test was carried out.

The significance difference between groups in the expression of the Type I collagen gene was considered to be P-value < 0.0001. The results of the Tukey Supplementary Test and group comparison are presented in Table 1. Changes in gene expression are significant if P <0.05. The results of the Tukey test are shown in Table 1. In this table, the intensity of decrease or increase in gene expression and the level of significance within the group were determined. The results of this test were evaluated at a significance level of P < 0.05. The results showed that the expression of the Type I collagen gene in the treatment groups was significantly increased compared to the model group. Comparing the groups showed that gene expression in aloe vera, aloe vera gelatin nanofibers, and aloe vera gelatin enriched nanofibers treatment group significantly increase compared to the control group. Also, no significant difference was observed between the treatment group of nanofibers and aloe vera gel. The comparison of gel treatment and enriched nanofibers indicates a significant difference.

In Figure 4, the model group was used as a reference for calculating fold change. According to the Figure 4, the expression level of the Type III collagen gene in all treatment groups is more than 1 compared to the model group. It indicates a significant increase in Type III collagen gene expression in the treatment groups compared to the Model group (reference). To evaluate and analyze the differences in gene expression between groups, an ANOVA test was carried out.

The significance difference between groups in the expression of the Type III collagen gene was considered to be P-value < 0.0001. The results of the Tukey Supplementary Test and group comparison are presented in Table 2. Changes in gene expression are significant if P <0.05. The results of the Tukey test are shown in Table 2. In this table, the intensity of decrease or increase in gene expression and the level of significance within the group were determined. The results of this test were evaluated at a significance level of P < 0.05. The results showed that the expression of the Type III collagen gene in the treatment groups was significantly increased compared to the model group. Comparing the groups showed that gene expression in aloe vera, aloe vera gelatin nanofibers, and aloe vera gelatin enriched nanofibers treatment group significantly increase compared to the control group. Also, no significant difference was observed between the treatment group of nanofibers and aloe vera gel. The comparison of nanofiber treatment and enriched nanofibers indicates a significant difference.

Discussion

In the present study, the role of aloe vera gelatin nanofibers enriched with *Arnebia euchroma* root hexane extract on the expression of the Type I, III collagen genes was investigated. The results showed that Extract, aloe vera gel, aloe vera gelatin nanofibers, and enriched nanofibers affect the expression of the Type I,III collagen genes after injury. The increase in gene expression was significant in the treatment group compared to the model group. However, in the in-group comparison and Tukey test, it was found that the increase in Type I,III collagen genes expression was statistically significant in the treatment group (Extract, gel, nanofiber, and enriched nanofibers) compared to the model group. Also, there was no significant difference between the nanofibers and aloe vera gel treatment group. The remarkable properties of nanofibers are the reason for using nanofibers. The nanofibers inhibit the aerosol particles with the trapping mechanism, provide a suitable pattern for vapor transfer, and prevent bacteria from penetrating the wound surface. Since these fibers have a high surface area, they are important in drug delivery [5]. The basic mechanism in microneedling is fine punches that cause controlled injury to the skin surface without damaging the epidermis. Little superficial bleeding occurs in these very small injuries. The wound healing mechanism occurs with the secretion of growth factors such as platelet-derived growth factor (PGF), Transforming growth factor-alpha (TGFα), Transforming growth factor-beta (TGFβ),

connective tissue activating protein, connective tissue growth factor, and Fibroblast growth factor (FGF) [12].

Tiny needles by destroying old filaments provide the conditions for the migration and proliferation of fibroblasts in the layers of the intercellular matrix, vascular regeneration, and new collagen formation [13, 14]. Lieb et al. proposed another hypothesis to explain microneedling [16]. The electrical potential of the membrane is -70 mv. When the needles are placed near the membrane, the electrical potential is increased to -100 mv. As a result, it induces Type I collagen fibroblast by increasing the activity of the cell and the release of various proteins, potassium, and growth factors from the cells. Thus, needles do not cause real wounds [14, 16, 17]. The expression of matrix metalloproteinases due to microneedling is believed to reduce hyperpigmentation. In addition, microneedling promotes keratinocytes and balances cells in acne.[16] Wound healing and deposition of new collagen lead to skin firmness and filling of atrophic wounds. In laboratory models, microneedling has increased the penetration of drugs through the skin barrier [18, 19]. Inflammation forms after microneedling. Shikonin in Arnebia euchroma hexane extract show their anti-inflammatory effect by direct inhibition of inducible nitric oxide synthase (iNOs) [20], inhibition of kappa-light-chain-enhancer of NF-κB (Nuclear Factor: activated B cells) [21], and inhibition of the activity pf MAPKs (Mitogen-activate Protein kinase) [22-25] Injury and cellular stress also increase the level of free radicals and cell death signals.[26] Reactive oxygen species (ROS) [27] attack vital macromolecules such as proteins, lipids, and fatty acids, causing oxidative stress damage. Studies have shown that Shikonin in hexane extract can scavenge oxygen free radicals [9]

Aloe vera has been introduced as one of the medicinal plants in wound healing. The anti-bacterial, anti-inflammatory, and anti-viral properties and angiogenesis of aloe vera extract have been proven [15, 28, 29] Numerous studies have confirmed the role of aloe vera products (extracts, gels, powders, oral solutions) on wound healing [30] Aloe vera contains glucomannan polysaccharides and is rich in mannose. Glucomannan affects fibroblast growth factor receptors and stimulates the activity and proliferation of these cells, leading to an increase in collagen production and secretion. Aloe vera mucilage not only increases the amount of collagen at the site of injury but also increases the cross-links of these fibers by altering the structure of the collagen, resulting in better quality collagen [31].

Insulin-like growth factors in normal skin are secreted by a small number of the dermis and epidermal cells. Moreover, it is secreted by most epidermal cells (macrophages and platelets) during skin damage. This family of growth factors stimulates mitogenic fibroblasts and is involved in the process of angiogenesis.[32] Other studies have shown that IGFs, along with other factors such as platelet-derived growth factor (PDGF), play an important role in wound healing and thus increase the thickness of the dermis and epidermis. In the basal layer of the normal skin epidermis, IGF gene expression was low. Nevertheless, this rate increases significantly in one or three days after the onset of ulcers [33, 34] Other studies have shown that an abnormal increase in IGF gene expression increases the expression and production of pro-alpha I from Type I collagen and pro-alpha I from Type II collagen in damaged fibroblasts, thereby increasing the wound effect [22, 35, 36]

Numerous studies have been performed on the use of synthetic compounds to control and inactivate factors involved in inflammatory and cancer pathways [16].

In the present research, aloe vera gelatin nanofibers were used as a treatment and drug delivery agent for *Arnebia euchroma* hexane extract. The nanofibers inhibit aerosol particles, by the trapping mechanism, provide a good pattern for vapor transfer and prevent bacteria from penetrating the wound surface. These fibers can release gradually in targeted delivery and drug delivery because of their high surface area [29]. This feature makes the dose of medicine smart.

Growth factors affect the signaling pathway of mitogen-activating protein kinase (MAPK). This path is related to growth factors. MAPKs are involved in cellular responses to stimuli such as mitogens, osmotic stress, heat shock, and pro-

inflammatory factors. They regulate cell function, including proliferation, gene expression, differentiation, cell survival, and apoptosis.

To bind the ligand, dimerization is needed. In this case, tyrosine kinase is activated and phosphorylation is performed. Protein receptor called GRB2It is activated through phosphorylation. This protein acts as a binder and activates another protein called SOS. Another protein called Ras is activated by activation of SOS. Activation of Ras, in turn, activates another protein called Raf. Raf also activates Raf kinases and then the cascade of kinases is activated. Finally, some genes including Type I collagen is expressed [37].

Conclusion

In the present research, controlled lesions were created on the skin of the animals during microneedling. The results showed that 48 h of treatment with aloe vera, aloe vera nanofibers, and enriched aloe vera nanofibers with Arnebia euchroma hexane extract significantly increased expression of Type I collagen and type III collagen genes at the wound site.

Aloe vera gelatin nanofibers and enriched aloe vera gelatin nanofibers, compared to aloe vera gel, can inhibit aerosol particles and vapor transfer. Moreover, they have a suitable surface for drug delivery and antibacterial and anti-inflammatory properties. Therefore, aloe vera gelatin nanofibers enriched with Arnebia euchroma hexane extract can be a part of a suitable dressing for repairing and rejuvenating the skin of male mice after microneedling.

Shikonin and glumanan in enriched nanofibers are gradually released and induce collagen I and collagen III genes expression and inhibit inflammation by passing through skin pores.

Certainly, Type I collagen and type III collagen genes expression and ECM repair are essential in skin rejuvenation.

Acknowledgments

We are very grateful for guidance and support of the Tehran North Branch, Islamic Azad University and thanks for publishing my article in Journal of Positive School Psychology.

Conflict of Interest Statement

The authors declare that no conflict of interest for this study.

Author Contributions Statement

Asadolah Shahrani: Concept, design, definition of intellectual content, literature search, clinical studies, experimental studies, data acquisition, data analysis, statistical analysis, manuscript preparation, manuscript editing and manuscript review; Maryam Bananeg: Concept and design, definition of intellectual content, literature searchdata acquisition, editing and manuscript review; Parvin Mansouri: Concept and design, definition of intellectual content, clinical and experimental studies; Hengameh Alibeik: Concept and design, data analysis, statistical analysis; Nahid Nickhah: Clinical and experimental studies, manuscript editing and manuscript review.

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Tables

Table 1. Tukey test results of collagen I gene expression.

Tukey.test	**Summary**	**p.value**	**Description**
Extract Vs. Model	****	<0.0001	2.4844fold
Gel Vs. Model	****	<0.0001	3.4452fold
NanoFibre Vs. Model	****	<0.0001	4.0699fold
NanoFibre+Extract Vs. Model	****	<0.0001	5.361fold
Gel Vs. Extract	*	<0.0473	1.3867fold
NanoFibre Vs. Extract	**	<0.0012	1.6382fold
NanoFibre+Extract Vs. Extract	****	<0.0001	2.1578fold
NanoFibre Vs. Gel	ns	<0.5658	1.1813fold
NanoFibre+Extract Vs. Gel	**	<0.0041	1.5561fold
NanoFibre+Extract Vs. NanoFibre	ns	<0.1235	1.3172fold

Table 2. Tukey test results of collagen III gene expression

Tukey.test	Summary	p.value	description
Extract Vs. Model	****	<0.00000	4.4704fold
Gel Vs. Model	****	<0.00000	6.6557fold
NanoFibre Vs. Model	****	<0.00000	5.0953fold
NanoFibre+Extract Vs. Model	****	<0.00000	10.7003fold
Gel Vs. Extract	*	<0.02720	1.4888fold
NanoFibre Vs. Extract	ns	<0.82701	1.1398fold
NanoFibre+Extract Vs. Extract	****	<0.00000	2.3936fold
NanoFibre Vs. Gel	ns	<0.22930	-1.3062fold
NanoFibre+Extract Vs. Gel	**	<0.00626	1.6077fold
NanoFibre+Extract Vs. NanoFibre	****	<0.00003	2.1fold

Figures

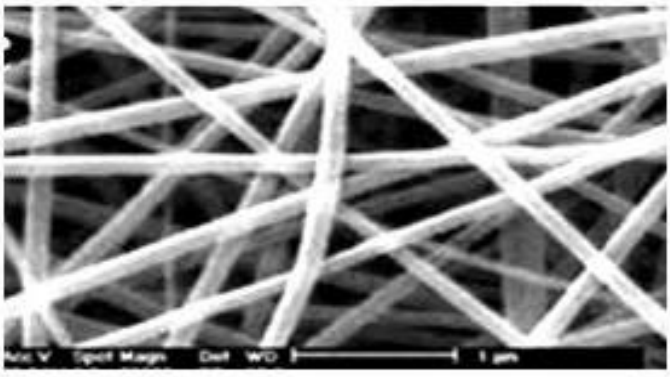

Figure 1.

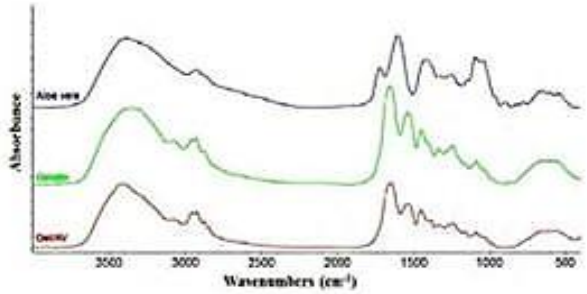

Figure 2.

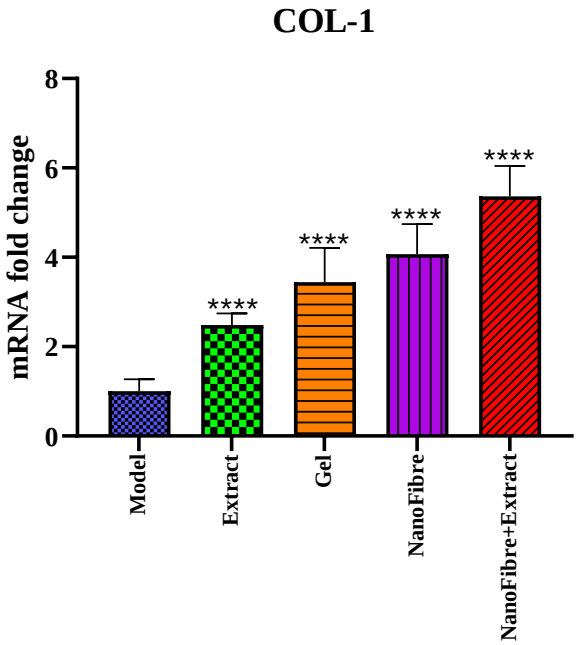

Figure 3.

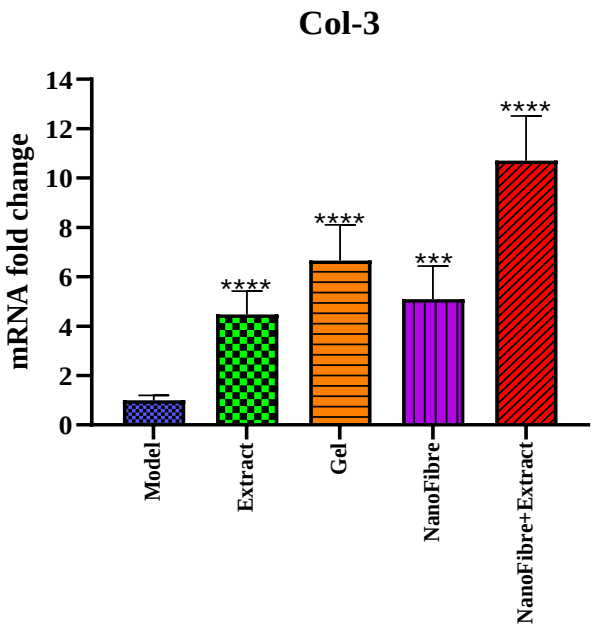

Figure 4.

Figure Legends

Fig 1. SEM image of gelatin/aloe vera nanofibers
Fig 2. FTIR Results
Fig 3. COL I gene expression changes in different treatments based on fold change
Fig 4. COL III gene expression changes in different treatments based on fold change