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Algae Glaucescens Sargassum enhances the expression of the FGF-7 gene, promoting wound healing

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Abstract

Introduction: Wound healing in the skin is a complex process with several cellular and molecular mechanisms. Chronic wounds such as diabetic ulcers and pressure ulcers are extremely difficult with their high frequency of recurrence and infectibility. Current studies have evidenced the potential of seaweed extracts in healing due to their antioxidant, anti-inflammatory, and regeneration properties. Sargassum Glaucescens , a brown alga, has been found to have the capability to accelerate cell regeneration. This study is intended to determine the specific impacts of Sargassum glaucescens extracts on FGF-7 gene expression, an important gene in wound healing.

Materials and Methods: Antioxidant activity of Sargassum glaucescens extract was determined by the DPPH inhibition assay. Human dermal fibroblast (HDF) cell lines were grown and exposed to various concentrations of the extract. The FGF-7 expression in treated cells was determined by using real-time PCR. HDF fibroblast cell line was developed and the toxicity of the extract was determined by the MTT assay

Results: Sargassum glaucescens extract was found to have high antioxidant activity with low IC50 value of 12 µg/ml. The toxicity of the extract was determined by the MTT assay, which indicated no toxicity. Upregulation of FGF-7 gene expression was achieved by treatment with the extract at 125 µg/ml, which was established by RNA extraction and real-time PCR.

Conclusion: Such findings indicate that the isolated algae of Sargassum glaucescens may be optimized as a drug to enhance wound healing activities

1. Introduction

Wounds can be caused by numerous reasons, including surgery, trauma, external causes (pressure, burns, and lacerations), or pathological conditions (diabetes, vascular diseases). Depending on their causes and consequences, these injuries are classified as acute or chronic wounds. Acute wounds tend to have a well-organized and appropriate healing process, and they lead to stable restoration of anatomical and functional integrity. Chronic wounds, however, are not able to achieve maximal anatomical and functional integrity(1). Wound healing is a function of the nature, severity, and state of pathological processes and of host and environmental state. Systemic factors such as patient's age, presence of vascular, metabolic, and autoimmune disease, and long -term medication can influence the process of wound healing(2).Epithelial cells of the skin are unstable elements that are continuously shed from the stratum corneum through the process of keratinocyte turnover and are replaced in the basal layer by differentiated elements originating

from the proliferation and differentiation of stem cells(3). The renewal of these cells varies depending on various factors such as trauma, hormonal influences, skin conditions, and the individual's health (4). This process involves multiple stages activated by intracellular and intercellular biochemical pathways, working in harmony to restore tissue integrity and homeostasis. Elements such as the coagulation cascade and inflammatory pathways are also involved. Various cells, including fibroblasts, keratinocytes, and endothelial cells, as well as neutrophils, monocytes, macrophages, lymphocytes, and dendritic cells, act as immune components in this process (5) reveals schematically the wound healing repair processes with different cells that are involved in each phase.

The formation of new tissue begins within two to ten days following the injury and involves cellular growth and migration of various cell types. If the injury is dermal, then a highly vascular and poorly differentiated connective tissue known as granulation tissue is formed(6), which has cellular and fibrous elements arranged in the form of an apparently amorphous matrix. The cells of the granulation tissue are composed of (i) fibroblasts responsible for the production of fibrous components; (ii) myofibroblasts that enable the process of wound contraction; and (iii) endothelial cells responsible for the process of neovascularization (7).

Wound healing of the skin is a multifaceted biological phenomenon that includes cellular interactions and several signaling pathways(8). Of the growth factors that are part of this process, fibroblast growth factor 7 (FGF-7), or keratinocyte growth factor (KGF), plays a crucial role in promoting keratinocyte proliferation and migration(9). These are critical phases in successful wound healing. Recent research has identified the ability of marine algae, in this case Sargassum glaucescens, to act as a reservoir for bioactive compounds that can initiate healing on wounds by modulating the expression of genes that encode the genes of this growth factor(10).

Tissue healing is regulated by the coordination of cell surface receptors and growth factors; the interaction promotes cell migration, induces angiogenesis, epithelialization, and matrix synthesis, and assists in reconstructing damaged tissue. Certain growth factor families are examined for their involvement in wound healing. One such family is FGF-7, a member of the Fibroblast Growth Factor (FGF) family(11).

FGF-7 or Keratinocyte Growth Factor (KGF) is a highly significant growth factor in wound healing that stimulates keratinocyte activity, angiogenesis, and extracellular matrix deposition through specific receptor binding and signaling. Its activity has a significant role in successful tissue repair and regeneration.

1-1 Sargassum glaucescens

Sargassum glaucescens, a brown seaweed belonging to the Sargassaceae family, is recognized for its diverse medicinal properties and has been utilized in traditional medicine across various cultures(12).

Sargassum glaucescens is a significant herbal medicine with various health advantages. It contains vital vitamins (A, C, E, and K), minerals (iodine, calcium, magnesium), and dietary fibers. These substances facilitate overall health and the sustenance of body functions (13). The presence of polyphenols and flavonoids in Sargassum glaucescens gives substantial antioxidant activity by neutralizing free radicals and thus reducing oxidative stress, which is associated with chronic diseases and aging (14). The Sargassum glaucescens extracts have been shown to contain anti-inflammatory activities, and thus would be useful in inflammative diseases like arthritis and skin disease. Sargassum glaucescens is antimicrobial against most bacteria and fungi and thus can be a potential natural drug applied to treat infection and skin diseases. Sargassum glaucescens' rich fiber nutrient ensures digestion and intestinal well-being, possibly curing constipation and improving the overall process of the digestive tract(15). . In most of the coastal villages, Sargassum glaucescens is employed in some traditional medication of various conditions such as respiratory problems, dermatitis, and as a food supplement(16). Sargassum glaucescens has been found to have bioactive compounds that improve wound healing mechanisms by favoring cell migration and growth, especially by increasing the activity of growth factors such as FGF-7(17).

This study investigated the effect of Sargassum glaucescens on the fibroblast growth factor 7 (FGF -7) gene expression, a significant in wound healing due to its stimulation of proliferation and migration

of keratinocytes. Since wound healing is a multi-faceted process encompassing more than a single cell interaction and a variety of signal transduction pathways, the present work seeks to elucidate how the bioactive constituents of Sargassum glaucescens can stimulate increased FGF-7 expression and therefore enhance effective tissue repair and regeneration. Through research on the therapeutic potential of the marine alga as a folklore wound healer, the study attempts to place relevant information in the limelight with regard to its medicinal significance and promote its utilization in traditional medicine for healing skin wounds and ailments.

2. Methods and Materials

2-1 Extraction by Maceration Technique

The Sargassum glaucescens was purchased from Chabahar fisheries and cleaned before powdering. Dry powder (0.5 kg) was stored in an oven at 45°C overnight. It was incubated in water at room temperature for 72 hours and then filtered with Whatman filter paper (No. 1).

2-2 Evaluation of Antioxidant Activity

Sargassum glaucescens extract free radical scavenging ability was evaluated through DPPH method. Here, extract and DPPH (Sigma Chemical Co., USA) were stock solutions of final concentration 10 mg/mL in reaction mixture. Hence, 50 µL of Sargassum glaucescens and 150 µL of DPPH solution were blended properly in a 96-well microtiter plate. Dimethyl sulfoxide (DMSO) was used as control. The reaction mixture was allowed to incubate for 30 minutes and was measured for the absorbance at a wavelength of 517 nm using a microplate reader (Thermo Electron Corporation, Finland). All of the measurements were performed in triplicate to estimate the accuracy and the values for absorbance were recorded accordingly.

2-3 Cell Culture

In the present study, the HDF cell line was obtained from Iran's Genetic and Biological Resources Center and was grown according to the proper protocol. DMEM culture medium was applied for cell preservation, supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin antibiotic combination at 37 degrees Celsius with a humid atmosphere of 5% CO2.

Periodically, the morphological status, viability, and cell count were observed under an inverted microscope. When the cells had reached over 70% of the flask surface, they were dislodged from the flask with 0.25% trypsin and centrifuged for 10 minutes at a speed of 1500 RPM. Then a cell suspension was prepared and cell viability was observed under a light microscope. After ascertaining that there was no contamination, the cells were prepared for further experiments.

2-4 Cytotoxicity Assessment

To check for the inhibitory activity of Sargassum glaucescens against HDF cell growth, the MTT assay was carried out. 10,000 cells were incubated in a 96-well plate for 24 and 48 hours, and IC50 values were measured with the MTT test.

In the experiment, the plant extract was used at concentrations of 0 to 500 µg/mL for 24 and 48 hours. The cells were subsequently incubated after adding 20 µL of MTT solution (0.5 mg/mL) to every well, where they were further incubated for another 4 hours at 37 degrees Celsius under a 5% CO2 environment.

Subsequently, the MTT solution was removed, and the formazan crystals that had been generated by viable cells were dissolved in 150 µL of dimethyl sulfoxide (DMSO). Finally, optical absorbance at 570 nm was measured in an ELISA reader, and cell viability percent was calculated.

2-5 Gene Expression Analysis

2-5-1 RNA Isolation from Cells

Following incubation for a specific time, normal cells were exposed to the IC50 concentration of Sargassum glaucescens extract in 6-well plates. Trizol protocol was followed for extraction of RNA according to the standard protocol. Chloroform and isopropanol were utilized for RNA separation and 75% ethanol was utilized for washing RNA. RNA quantity and integrity were determined after extraction by a Nanodrop machine and agarose gel electrophoresis.

2-6 Quantitative and Qualitative RNA Analysis

2-6-1 Quantitative Analysis Using Nanodrop

For quantitative RNA analysis of the isolated RNA, Nanodrop was utilized. For that, a tiny sample of the RNA sample (generally 1 to 2 µL) was

applied on the sensor of Nanodrop. The RNA in 260 nm was measured for the concentration and absorbance 280/260 nm ratio was conducted to determine the purity of RNA. When there is the usage of a ratio of 1.8 or more, there will be good quality as well as good purity of RNA. These readings are required in order to determine that the RNA obtained is in a form which is suitable for subsequent experiment stages, i.e., PCR reactions and cDNA synthesis.

2-6-2 Qualitative RNA Analysis by Electrophoresis

RNA, purified, was subjected to electrophoresis using 1% agarose gel and viewed on a gel documentation system, with clear bands of RNA.

Here, 0.25 g agarose powder was dissolved in 25 mL 1X TBE buffer in a microwave oven until the solution turned clear. The solution was then added with 0.5 µL Red Dye and cast into the gel tray over which a comb was positioned inside. At 10 minutes, 5 µL of each of all samples was loaded into wells (since the samples were RNA, they were preceded by blending them with 1 µL Blue Dye) and a power supply of 110 volts was used. At 20 minutes, the gel was submerged in the gel documentation system to make the bands visible under UV. Having ensured integrity of RNA extracted, photographs of the gel were taken.

2-7 cDNA Synthesis

Equal quantities of RNA were taken for the synthesis of cDNA. cDNA synthesis was done using RevertAid RT Transcription Kit from Thermo Fisher following the standard protocol (as shown in Table 3-3). Since DNA polymerase lacks any capability to use RNA as a template directly, DNA is initially synthesized by a strand of DNA from RNA.

For this, the reverse transcriptase enzyme was used for cDNA synthesis by transcribing RNA as per instructions. Reverse transcription was done with random hexamers. Synthesized cDNA was kept in a freezer at -70 degrees Celsius. Microtubes were filled on a thermocycler as per the thermal cycling table, and lastly, samples synthesized were kept at -20 degrees Celsius for use in further experiments.

2-8 PCR Setup with Primers

PCRs were performed with GAPDH-specific primers as the housekeeping gene to cross-compare the designed primers and treated and untreated synthesized cDNA to validate them. The nucleotide sequences of these primers are listed in Table 1.

Further, the size and specificity of primers that were ordered were confirmed using the BLAST program and aligned onto the target genome to amplify effectively. This is a very important step to confirm that the primers will hybridize specifically with the target sequences to PCR.

2-9 Real-Time PCR Setup

HDF cells were first seeded into a 6-well plate for Real-time PCR preparation and incubated independently with diluted concentrations of the synthesized sarcosome extract for 48 hours. FGF7 gene expression was then measured by the SYBR Green method following RNA isolation and cDNA synthesis. qRT-PCR reaction mixture was prepared to a final volume of 20 µL as: 10 µL SYBR Green (2X), 2 µL of reverse primer and 2 µL of forward primer each of 10 µM concentration, 7 µL of deionized water, and 1 µL of cDNA with GAPDH as an internal control. The Bioneer Exicycler 96 system was optimized with the initial denaturation of 95°C for 10 minutes and then 40 cycles of 95°C for 20 seconds of denaturation, 65°C for 20 seconds of denaturation, 65°C for 20 seconds of annealing, and 72°C for 20 seconds of extension. Gene expression was calculated by the ΔΔCt method.

3. Result

3-1 Extraction and Antioxidant Capacity Determination

Extraction was done via the maceration method. Measurement of the plant extract's antioxidant activity was done via the DPPH method. Sargassum glaucescens extract was specifically measured in terms of antioxidant activity.

Antioxidant capacity of Sargassum glaucescens extract was determined and had an IC50 of 12 mg/ml as indicated by Table 2. The above was contrasted with that of quercetin with an IC50 of 0.005 µg/ml. This puts the potency of quercetin as an antioxidant into perspective against the extract (Fig 1).

Table 1: Nucleotide Sequences of Primers

Primer	Sequence (5'to3')	Length	Lenght	GC%	Tm° C
FGF7	Forward	CTGTCGAACACAGTGGTACCTG	22	60.86	54.55
	Reverse	CCAACTGCCACTGTCCTGATTTC	23	61.92	52.17
GAPDH	Forward	TGCCTCCTGCACCACCAAC	19	63.16	62.79
	Reverse	CGGAGGGGCCATCCACAG	18	72.22	62.18

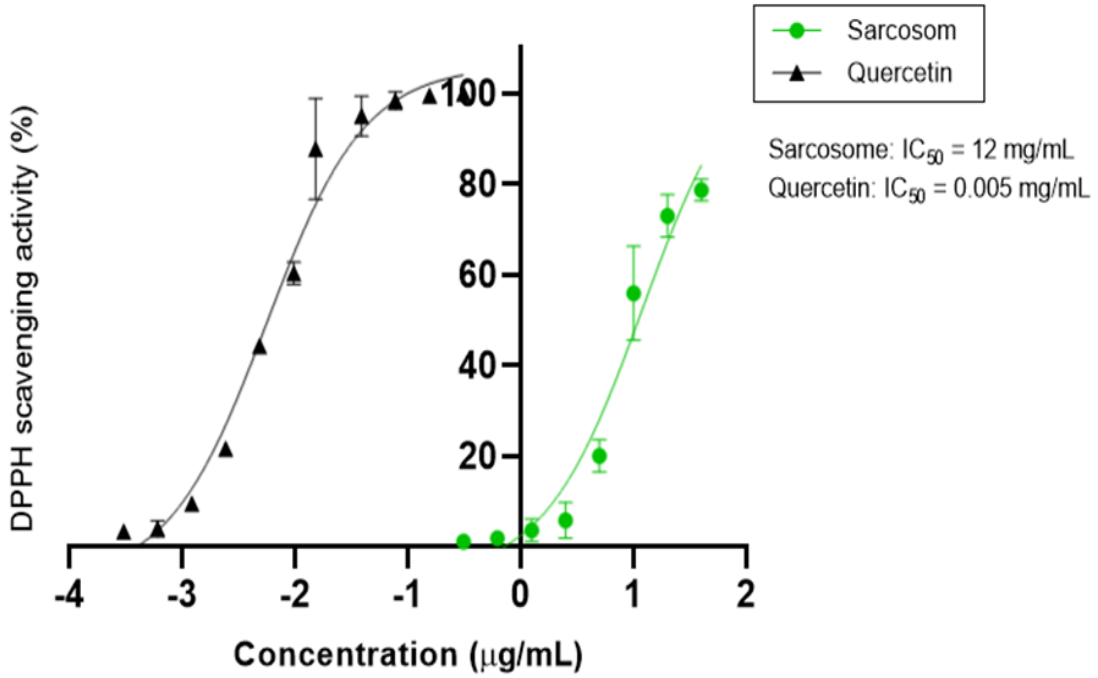

Figure 1: Antioxidant Results of Sargassum glaucescens Extract

Table 2. Antioxidant Results of Sargassum glaucescens Extract

Extract	IC50(mg/mL
Sargassum glaucescens Extract	12 (mg/ml)
Quercetin Extract	0.0056 (mg/ml)

3-2 Cytotoxicity Assays using MTT

Human HDF (human dermal fibroblast) cells were treated with differing concentrations (0, 31.2, 62.5, 125, 250, and 500 µg/mL) of Sargassum glaucescens plant extract. The effect of this extract on HDF cell growth was evaluated at 24 and 48 hours. The results were that:

After 24 hours of treatment, Sargassum glaucescens extract-treated HDF cell viability was at 96% in comparison to the untreated control.

At 48 hours of treatment, the viability of HDF cells was 92% relative to untreated control.

These results show that the Sargassum glaucescens extract was non-cytotoxic to the HDF cell line. The extract increased the cell viability of HDF cells when compared to the untreated control.

Therefore, Sargassum glaucescens extract could be considered tolerable and non-toxic when it is in contact with HDF cells even at the maximum concentration under test (500 µg/mL). This shows the potential for conducting further studies on this extract for application with human dermal fibroblasts (Fig 2).

3-3 How Sargassum glaucescens Extract Affects HDF Cells

We wondered what would happen to HDF (human dermal fibroblast) cells if we treated them with Sargassum glaucescens extract, so we observed them under a microscope after two days of treatment. This is what we observed:

* HDF cells that received 125 mg/mL of Sargassum glaucescens extract grew bigger than the untreated ones.

* The HDF cells became more elongated.

* In addition, there were more HDF cells packed together compared to the group that was not treated.

Thus, it seems that Sargassum glaucescens extract is good for HDF cells. The cells became longer and more elongated, and there were more of them. That means they're alive and growing rapidly. This change in the shape of the cells and the fact that their number increased shows that the plant extract has the potential to make human skin cells grow and carry out their functions. This would be greatly beneficial in tissue or cosmetic production. (fig 3).

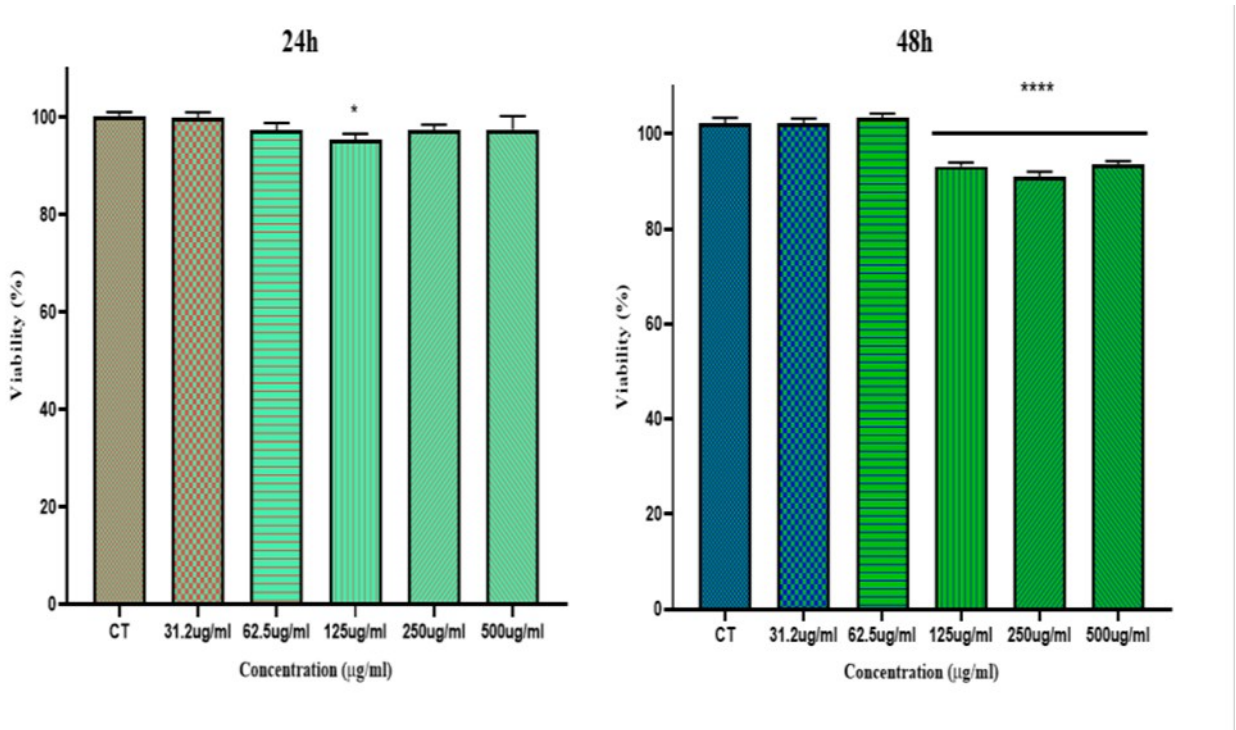

Figure 2: Cell Viability Results of HDF Cells Treated with Different Concentrations of Sargassum glaucescens Extract

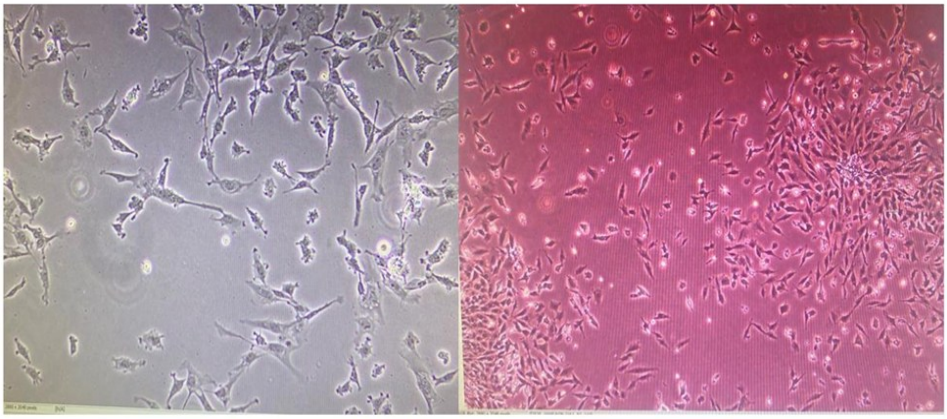

Figure 3: Phenotype of Cells After Treatment with Sargassum glaucescens. Left image: Before treatment, Right image: After treatment

3-4 FGF-7 gene expression

3-4-1 Quality Assessment of RNA Extraction

After the extraction of RNA, the quality of the extracted RNA was evaluated using agarose gel electrophoresis on a 1% agarose gel. The results showed the presence of two distinct bands corresponding to the 18S and 28S ribosomal RNA subunits (Fig 4). The observation of these two clear bands is an indication of the high quality and integrity of the extracted RNA sample. The presence of distinct 18S and 28S rRNA bands is a hallmark of intact, high-quality RNA, as these ribosomal RNA subunits are the most abundant RNA species in cells.

3-4-2 Quantification of Extracted RNA

The concentration and optical density (OD) of the RNA samples were measured using a NanoDrop spectrophotometer.

To assess the quantity of the extracted RNA, the optical absorbance of the RNA solution was measured at wavelengths of 260 nm and 280 nm. The results are presented in Table 3.

The results demonstrate that the RNA extraction method used in this study was effective in isolating high-quality and high-quantity RNA from both the control and treated cell samples. The concentration and purity of the extracted RNA are within the acceptable range, indicating that the samples are suitable for further downstream applications, such as gene expression analysis or RNA sequencing.

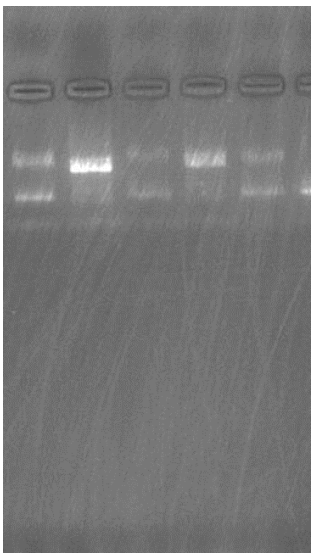

Figure 4: RNA extracted from cells

Table 3: Concentration and 260/280 absorbance ratios for selected RNA samples

Sample	Concentration (µg/mL)	OD 260/280
Control cells (untreated)	428	1.82
Treated cells	523	1.93

3-5 Gene Expression Analysis of FGF7

To investigate the expression of the FGF7 genes, real-time PCR (RT-PCR) analysis was used. The amplification curve was obtained by measuring the changes in fluorescence levels using the Real-Time PCR instrument, which shows the amplification of the samples. Figure 6. shows the amplification curve, and Figure 5-9 shows the melting curve for the FGF7 genes.

The comparison of the expression of FGF7 with GAPDH shows that the expression level of FGF7 is 1.952 times higher in the sample. The p-value (H1) is equal to 0, which indicates a statistically significant difference between the two groups (Fig 7).

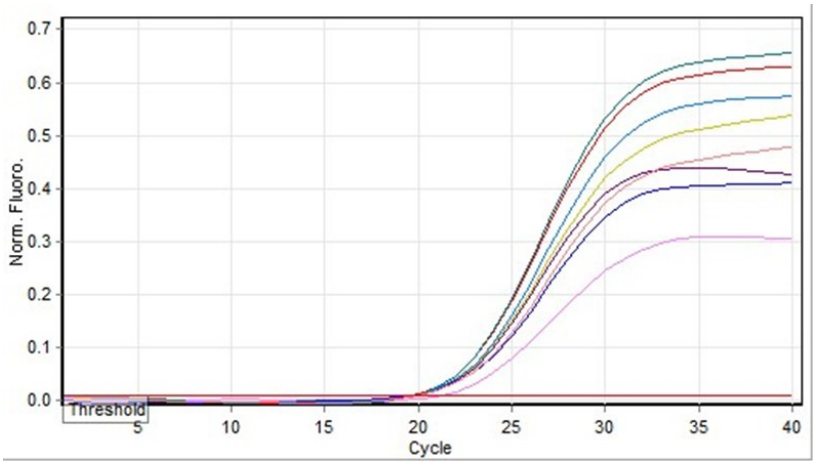

Figure 5: shows the amplification curve, genes.

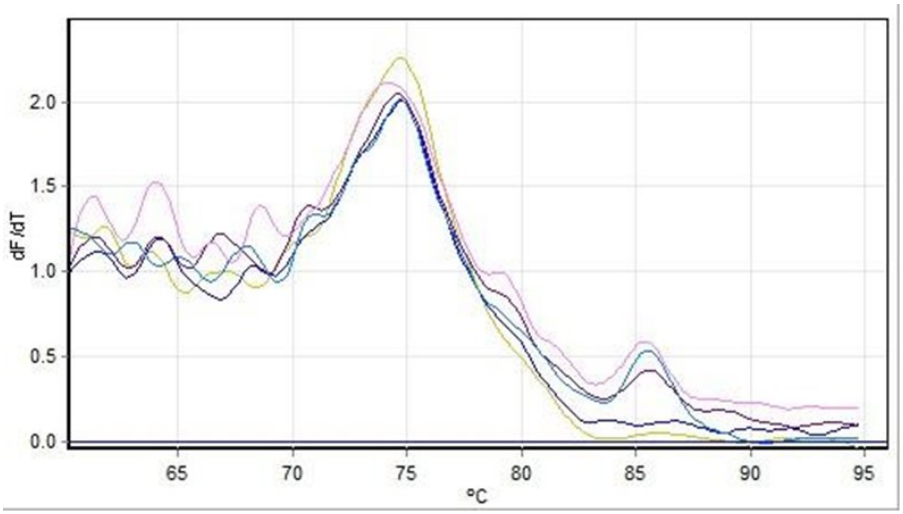

Figure 6: shows the melting curve for the FGF7

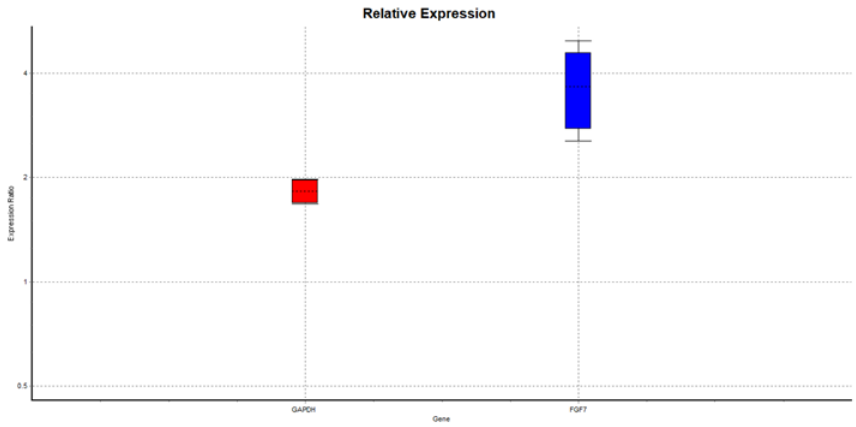

Figure 7: The comparison of the expression of FGF7 with GAPDH

4. Discussion

The findings of the current study contribute to the growing body of evidence on the therapeutic potential of marine algae, and indeed Sargassum species, in skin wellness and wound healing. Other researchers have documented anti-inflammatory, antioxidant, and skin repair activities of marine algae in support of our findings. For example, the potent analgesic and anti-inflammatory properties of Sargassum swartzii, as reported by Hong Diem Dang et al., are consistent with our findings of high cell viability and morphological enhancements in Sargassum glaucescens-treated human dermal fibroblasts (HDF)(18).

Our results demonstrate that Sargassum glaucescens exhibits a notable antioxidant capacity, with an IC50 value of 12 mg/mL, which is considerably higher than that of quercetin (0.005 µg/mL) (18). This suggests that while Sargassum glaucescens is a potent antioxidant, it may require higher concentrations to match the efficacy of established antioxidants like quercetin. The ability of Sargassum glaucescens to suppress reactive oxygen species (ROS) production and enhance cellular proliferation supports its role in mitigating oxidative stress, a crucial factor in skin aging and wound healing(19).

The stimulation of fibroblast proliferation and keratinocyte activity by Sargassum glaucescens is particularly significant. Our findings indicate that the extract promotes cell growth and enhances the morphology of HDF cells, which is consistent with previous studies showing that marine algae can induce cell proliferation and support wound healing processes (20,21). The upregulation of FGF7 in treated HDF cells, as demonstrated by our qReal-Time PCR analysis, underscores the extract's potential to stimulate epithelialization and collagen synthesis, essential processes in effective wound healing (22).

The regulation of genes associated with skin barrier function and extracellular matrix components, such as filaggrin and keratins, further emphasizes the therapeutic potential of Sargassum glaucescens. These findings are in line with prior research highlighting the importance of these proteins in maintaining skin hydration and the barrier (23). By promoting the expression of these genes, Sargassum glaucescens may enhance the skin's natural defense mechanisms against environmental stressors.

Furthermore, the results of our study on the role of the TGF-β signaling pathway in wound healing are particularly relevant. The overexpression of genes involved in this pathway in tumor tissue, as outlined in recent literature (24), suggests that inhibition of TGF-β can provide new therapeutic strategies for skin restoration(25). Our findings are in agreement with this perspective, as modulation of expression of FGF7 indicates potential pathways through which Sargassum glaucescens could impact wound healing.

5. Conclusion

The significance of these results transcends science because they suggest that Sargassum glaucescens and other algae in the marine environment can be utilized to develop novel therapeutic solutions for skin conditions, including diabetic wounds and other impairments of healing.

In summary, our study highlights the significant therapeutic potential of Sargassum glaucescens in promoting skin health and enhancing wound healing. The extract demonstrated strong antioxidant properties and effectively stimulated the proliferation of human dermal fibroblasts and keratinocytes. Additionally, the upregulation of key genes, including FGF7, suggests a mechanism through which Sargassum glaucescens can facilitate epithelialization and collagen synthesis. These findings support the use of marine algae as a promising natural resource for developing innovative treatments for skin conditions and underscore the need for further research to fully explore their applications in dermatology.

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Conflict of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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