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In Vitro Effects of the Phytocomplex TrichoTech™ on Human Fibroblasts: Proliferative Potential and Effects on Gene Expression of FGF-7 and FGF-10

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Abstract

The human hair follicle, a mini-organ formed with neuroectodermal-mesodermal interaction, is a complex structure, in the active steady state (anagen) the dermal papilla can be considered as a ball of extracellular matrix, surrounding specialized fibroblasts. The cross-talk of dermal papilla with neighbouring matrix cells results in the maintenance of hair fibre production. This study aimed to investigate the proliferative potential of the compound Trichotech™, a phytocomplex obtained from a mixture of essential oils, on cultured human fibroblasts and its ability to modulate the gene expression of FGF-7 and FGF-10. Trichotech™ was shown to enhance fibroblasts proliferation in concentrations of 0.5% to 2.0%, and also increase the percentage of cells in the S/G2/M phases of the cell cycle. Trichotech™ at both 1.0% and 2.0% induced a statistically significant effect on wound healing assay compared to the untreated control. We examined the interaction between cell survival (PI3K/Akt) and mitogenic (Ras/MAPK) signal transduction pathways after Trichotech™ treatment (1.0% and 2.0%) on the fibroblast cell line. Trichotech™ caused phosphorylation of ERK1/2, as well as greater phosphorylation of MEK in comparison with both the untreated control and ERK1/2. PI3K and AKT, however, were not shown to be significantly more phosphorylated following Trichotech™ exposure. To verify the relative expression of

mRNA for FGF-7 and FGF-10 genes, a real-time polymerase chain reaction (qPCR) protocol was used. Results show the increase in mRNA expression by fibroblasts after treatment with Trichotech™. In both concentrations tested, Trichotech™ was found to increase the expression of FGF-7 and FGF-10. Sirius red staining allows for rapid assessment of collagen content, it showed a significant increase in collagen content in treated fibroblasts. Further investigation concerning Trichotech™ could be helpful towards the development of new bioactive phytocomplexes for dermatological and trichological use.

Keywords

Trichotech™, Fibroblasts, Proliferation, FGF-7, FGF-10

1. Introduction

The human hair follicle, a mini-organ formed with neuroectodermal-mesodermal interaction [1], is a complex structure consisting of an outer root sheath, an inner root sheath, the hair shaft, the bulge and the sebaceous gland [2].

The follicle undergoes successive steps of fibre production, regression and rest, which in humans last for an average of 3 years, 3 weeks and a few months, respectively. An additional phase involving the active release of the club fibre has also been described, and is thought to be independent from the rest of the hair cycle [3], while bearing no direct consequence on fibre production initiation [4].

Human hair follicle dynamics are regulated through a bi-stable equilibrium state, including an active steady state (the anagen stage) and a resting steady state (the telogen stage); the transition between these two steady states involves either a degradation phase (the catagen phase) or a neo-morphogenesis phase (the neogen phase). It is now believed that mesenchymal and epithelial oscillators control the stochastic autonomous switching between these two steady states [5].

In the active steady state (anagen), the dermal papilla can be considered as a ball of extracellular matrix, surrounding specialized fibroblasts. The cross-talk of dermal papilla with neighbouring matrix cells results in the maintenance of hair fibre production [6].

The dermal papilla maintains bulge stem cells and secondary hair germ cells quiescent during telogen through production of bone morphogenetic protein 4 (BMP4) and fibroblast growth factor 18 (FGF-18). Cell proliferation during anagen is triggered via production of BMP inhibitors (e.g. Sosrdc1 and Bmbi), as well as secretion of FGF-7 and FGF-10 [7] [8]. Thus, a combination of factors secreted by dermal papilla fibroblasts generates a signaling environment that dictates whether hair follicles will remain dormant or enter the anagen stage.

In a recent study, [9] found that topical application of FGF-10 was able to induce significant hair growth in C57BL/6 mice, linked to induction of anagen phase and increase in the number of hair follicles due, at least in part, to upre-

gulation of β-catenin and Shh signaling.

The use of certain phytocompounds as stimulants of hair growth has been considered an effective secondary measure for the treatment of hair loss, especially when common first-line treatments such as minoxidil application or finasteride administration yield poor results or cause adverse reactions. Many plant extracts and fractions thereof have been shown to elicit hair growth in mice [10] [11] [12], thus the prospecting of plant extracts as a source of hair growth-promoting compounds is a promising strategy.

The present study aimed to investigate the proliferative potential of the compound Trichotech™, a phytocomplex obtained from a mixture of essential oils, on cultured human fibroblasts and its ability to modulate the gene expression of FGF-7 and FGF-10, as well as to propose further applications on hair growth.

2. Materials and Methods

2.1. Chemicals

Propidium iodide, Direct Red 80 (Sirius Red) and 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide (MTT) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The AnnexinV/FITC Apoptosis Detection Kit was obtained from BD Pharmigen (CA, USA). Iscove's Modified Dulbecco's Medium (IMDM) and all cell culture reagents were purchased from Life Technologies (Thermo Fisher Scientific, USA). MEK, ERK, PI3K and AKT primary antibodies and Alexa Fluor 488-conjugate monoclonal antibodies were acquired from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

2.2. Cell Culture

CCD-1072Sk (ATCC® CRL2088™) fibroblasts were cultured in ISCOVE'S medium with 10% fetal bovine serum, 0.292 g/l L-glutamine, 1.0 g/l D-glucose, 2.2 g/l $NaHCO_3$, 10.000 UI penicillin, and 0.060 g/l streptomycin. Cells were kept in 25 cm^2 flasks (1 × 105 cells/ml) in a humidified incubator at 37˚C with an atmosphere of 5% CO_2 for a maximum of 30 population doublings. In all experiments, the fibroblast cultures were subjected to cell viability assays using Trypan blue dye, and readings were performed in a hemocytometric chamber under a light microscope. All experiments described were performed when cell viability was equal or above 95%.

2.3. MTT Reduction Cell Viability Assay

The MTT reduction assay ([3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide]) is employed with great success for estimating the number of viable cells in initial drug screenings. Its interpretation serves as an indicative of cellular metabolic activity, and the redox reactions occur in both the mitochondria and the cytosol. The reduction of the MTT salt to formazan happens mainly because of the succinate dehydrogenase enzyme, and results in purple insoluble formazan crystals. The intensity of the color is used to measure mitochondrial activity and, therefore, cell viability [13]. Cells were seeded at a density of 5×10^4

cells/well and treated with different concentrations of Trichotech™ (0.5, 1.0, 1.5 and 2.0%) for 24 hours. Next, 10 µl of a 5 mg/ml MTT solution (Sigma-Aldrich) were added to each well. After 4 hours the samples was reincubated with 100 µl of Sodium dodecyl sulfate (SDS) solution [10%] for 12 hours, and then optical density was measured in a FlexStation® 3 multimode Benchtop Reader (Molecular Devices, CA, USA) at 540 nm.

2.4. Propidium Iodide (PI) Incorporation Assay

Propidium iodide incorporation assays were performed using flow cytometry to assess the cellular fraction in the S/G2/M phase of the cell cycle (*i.e.* proliferating cells). To summarize, cells were seeded in 24-well plates at an initial density of 2×10^5 cells/well, to which was added a hypotonic fluorochrome solution (HFS—0.1% w/v sodium citrate, 0.5% w/v Triton X-100 and 50 µg/ml propidium iodide). After an incubation period of 4 h at 4˚C and shielded from light, the cells and supernatant were collected and analyzed. A FACS can flow cytometer and the CellQuest software were employed, and the data obtained were analyzed with WinMDI 2.8, considering 20,000 events per analysis for each assay.

2.5. Wound Healing

For this assay, fibroblasts were seeded in 6-well microplates and cultured as described above until observation of a confluent monolayer. The cell monolayers were carefully "scratched" with a sterile pipette tip, and washed with saline and PBS to remove loose cells and debris. Next, the cells were incubated at 37˚C with culture medium without fetal bovine serum (nutrient deprivation) and with 0.2% low molecular weight HA. Reference points near the "wound" were demarcated to ensure the same area of image acquisition. Images were obtained at different times using a digital camera attached to the microscope, and the percentage of wound closure was calculated using IMAGEJ (NIH, USA).

2.6. Measurement of MEK/ERK and PI3K/AKT Signaling Activity in Fibroblasts

Protein phosphorylation is a dynamic process controlled by the enzymatic activities of kinases and phosphatases. In order to inhibit these processes rapidly, fixation was done by adding BD FACS™ Lysing Solution. Following fixation, the cells were pelleted by centrifugation, and cell permeabilization was carried out either by use of a saponin solution, for analysis of cytoplasmatic proteins, or Triton X-100 solution for nuclear proteins. Following fixation and permeabilization, the samples were washed once with PBS and processed for antibody labeling. Antibody dilutions were prepared using PBS with BSA and sodium azide, with primary antibody concentrations according to the manufacturer's instructions. Samples were incubated for 2 hours at room temperature for labeling. Then the samples were washed and incubated with the secondary antibody for 45 minutes also at room temperature. After labeling with the primary and secondary antibodies, samples were read at 100.000 events in a BD Accuri™ C6 flow cyto-

meter.

2.7. Real-Time PCR (qPCR)

Total RNA extracted from fibroblast samples was converted to cDNA using a SuperScript® III RT kit (Invitrogen, Carlsbad, CA). A qPCR analysis was performed in 10 µL reactions with the SYBR GREEN PCR Master Mix and analyzed on a StepOnePlus™ Real-Time PCR instrument (Invitrogen, Carlsbad, CA). Relative standard curves were generated by serial dilutions and all samples were run in triplicates. Primers used are: FGF-7 forward (5'-ATCAGGACAGTGGCAGT TGGA-3'); FGF-7 reverse (5'-AACATTTCCCCTCCGTTGTGT-3') and FGF-10 forward (5'-CACATTGTGCCTCAGCCTTTC-3'); FGF-10 reverse (5'-AGGTGA TTGTAGCTCCGCACA-3'). The PCR reaction was performed under the following conditions: 50˚C (2 min), 95˚C (10 min), and 40 cycles of 95˚C (15 s) and 55˚C (1 min). GAPDH was used as a control gene.

2.8. Sirius Red Collagen Quantification

After cells were cultured, the medium was removed and the wells were washed three times with 0.1 M PBS. Next, 100 µl of Bouin's solution (picric acid 0.9%, formaldehyde 9.0% and glacial acetic acid 5.0%) were added for fixation for 1 h. Samples were washed with PBS, then the Sirius Red dye was added. After 1 h, the maximum possible amount of dye was removed, followed by washing with 150 µl of a 0.01 M hydrochloric acid solution for 30 seconds to remove the dye that did not bind to collagen. Next, the dye was removed from cell layers by the addition of 0.1 M NaOH for 30 min. 100 µl aliquots of the solution contained in the wells were transferred to a new plate. Absorbance was measured with an Elx-800-UV (Bio-Tek Instruments, USA) microplate reader at 570 nm.

2.9. Statistical Analysis

Results were given as mean ± SEM (standard error of the mean). The results obtained were statistically analyzed using a one-way analysis of variance (ANOVA), followed by Tukey's test a posteriori. Semi-quantifications were analyzed using Student's t-test. P-values < 0.05 were considered significantly different. Analyses were performed using GraphPad Prism version 5.0 (GraphPad Software Inc., CA, USA).

3. Results and Discussion

3.1. MTT to Formazan Reduction Assay

The MTT colorimetric assay is an established method of determining viable cell number in proliferation and cytotoxicity studies. This colorimetric assay provides accurate and reliable quantification of viable cell number and is based on the cleavage of the yellow tetrazolium salt, MTT, to form a soluble blue formazan product by mitochondrial enzymes, and the amount of formazan produced is directly proportional to the number of living, not dead cells, present during

MTT exposure [14]. Trichotech™ was shown to enhance fibroblasts proliferation (Figure 1). This effect of Trichotech™ in concentrations of 0.5% to 2.0% was visible by MTT assay after 24 hours of treatment ($P < 0.05$). Trichotech™ at concentrations of 1.5% and 2.0% significantly enhanced the proliferation of fibroblasts compared to the untreated group. Notice a dose-dependent increase in fibroblast proliferation.

3.2. Propidium Iodide (PI) Incorporation Assay

Progression through the cell cycle is one of the most fundamental features of cells and can be measured by staining cells with propidium iodide (PI). The level of PI fluorescence in a cell is, directly proportional to the DNA content of that cell, the quantification of which indicates the percentage of cells in each phase of the cell cycle in a sample [15]. As seen in Figure 2, the percentage of cells in the S/G2/M phases of the cell cycle increased in a dose-dependent fashion upon Trichotech™ treatment. This shows that Trichotech™ acts in accordance with several lines of evidence which support a molecular mechanism in the response to stimulation by natural compounds, increasing G2/M phase in fibroblasts [16] [17].

3.3. Wound Healing

Cell migration and proliferation coupled with controlled cell cycle are beneficial for the repair of sagged and wrinkled skin, dermal, and gastrointestinal wound healing. The *in vitro* scratch assay is a well-developed method to measure cell migration and its steps involve creating a "scratch" in a cell monolayer, capturing the images at the beginning and at regular intervals during cell migration to close the scratch, and comparing the images to quantify the migration rate of the

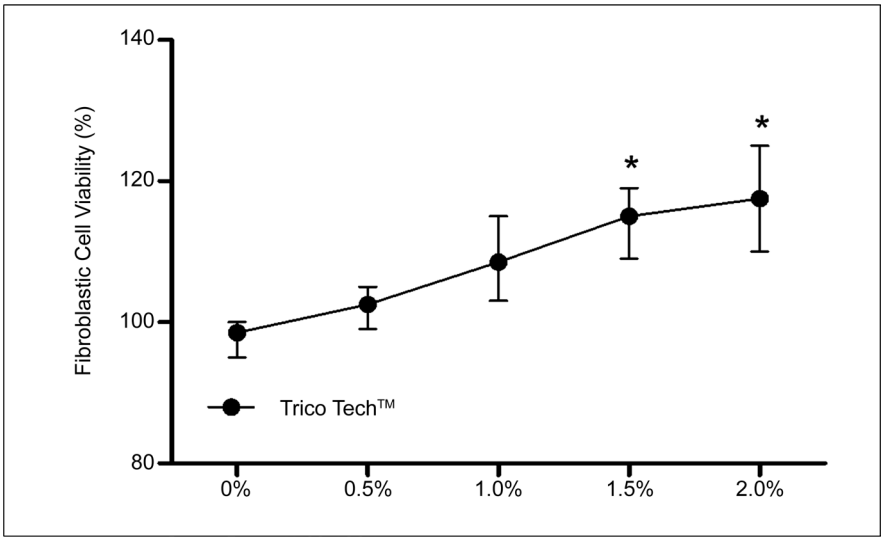

Figure 1. Results of cell proliferation from MTT reduction assay after 24-hour exposure to different concentrations of Tricotech™. Before starting the tests, cells were deprived of fetal bovine serum. (*) $P < 0.05$—significant in relation to control. ANOVA, Tukey. Assays performed in triplicate. GraphPad Prism v5.0.

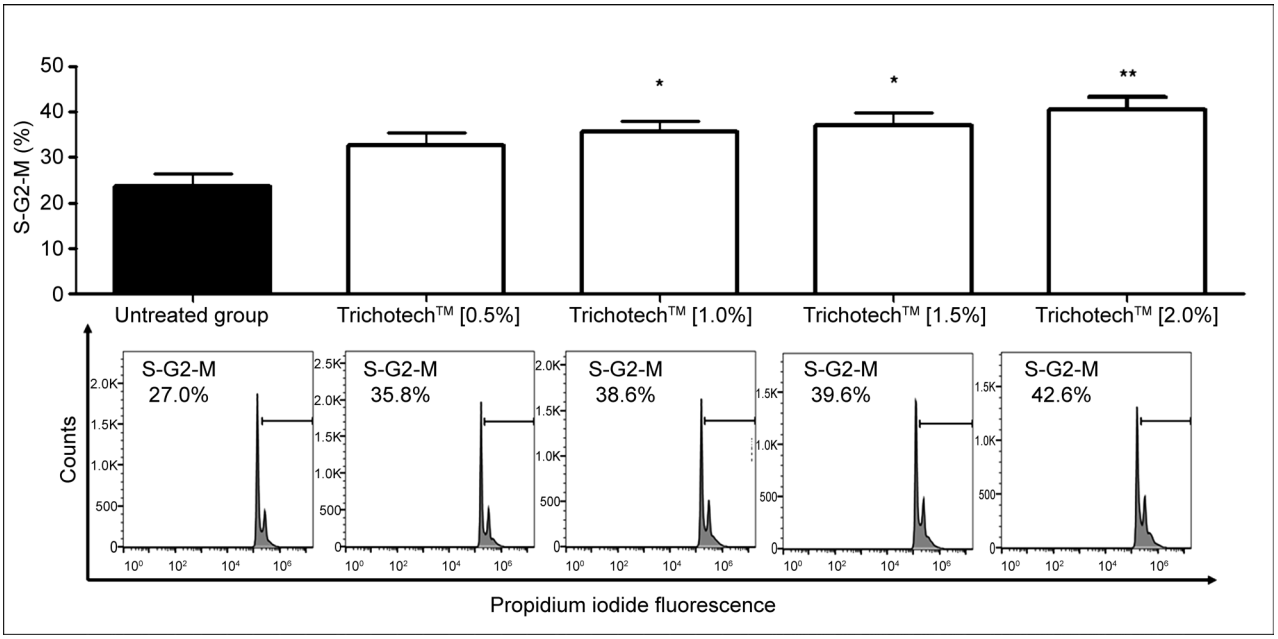

Figure 2. (a) Percentage of cells in S/G2/M phase obtained after 24-hour exposure of CCD-1072Sk cells to different concentrations of Tricotech™. Before starting the tests, cells were deprived of fetal bovine serum. (*) $P < 0.05$—significant in relation to control. ANOVA, Tukey. GraphPad Prism v5.0. (b) Histogram representing statistically significant concentration (10%). FlowJo v10.0.

cells [18]. We evaluated Trichotech™ wound healing stimulating activity on fibroblast cells using the scratch assay. Scratches were made on confluent fibroblast monolayers, which were then exposed to Trichotech™ for 24 h at two concentrations (1.0% and 2.0%). Trichotech™ at both 1.0% and 2.0% induced a statistically significant effect on wound closure compared to the untreated control (**Figure 3(b)**). In **Figure 3(a)** and **Figure 3(b)**, respectively, we can see images obtained at different times and the graphic representation of the distance between the edges of the scratch.

3.4. Measurement of MEK/ERK and PI3K/AKT Signaling Activity in Fibroblasts

An understanding of the mechanisms that regulate the cell migration and proliferation of dermal fibroblast cells by a natural compound could be beneficial in devising novel therapies to regulate fibrosis and wound contraction to ultimately improve the wound healing process [19]. The most highly studied intracellular signaling cascades in the context of cancer are the mitogen activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K)/AKT pathways [20]. We examined the interaction between cell survival (PI3K/Akt) and mitogenic (Ras/MAPK) signal transduction pathways after Trichotech™ treatment on the fibroblast cell line. The cells were stimulated with 1.0% and 2.0% Trichotech™. The treatment with Trichotech™ caused phosphorylation of ERK1/2, as well as greater phosphorylation of MEK in comparison with both the untreated control and ERK1/2 (**Figure 4**). PI3K and AKT, however, were not shown to be significantly more phosphorylated following Trichotech™ exposure. Similar results

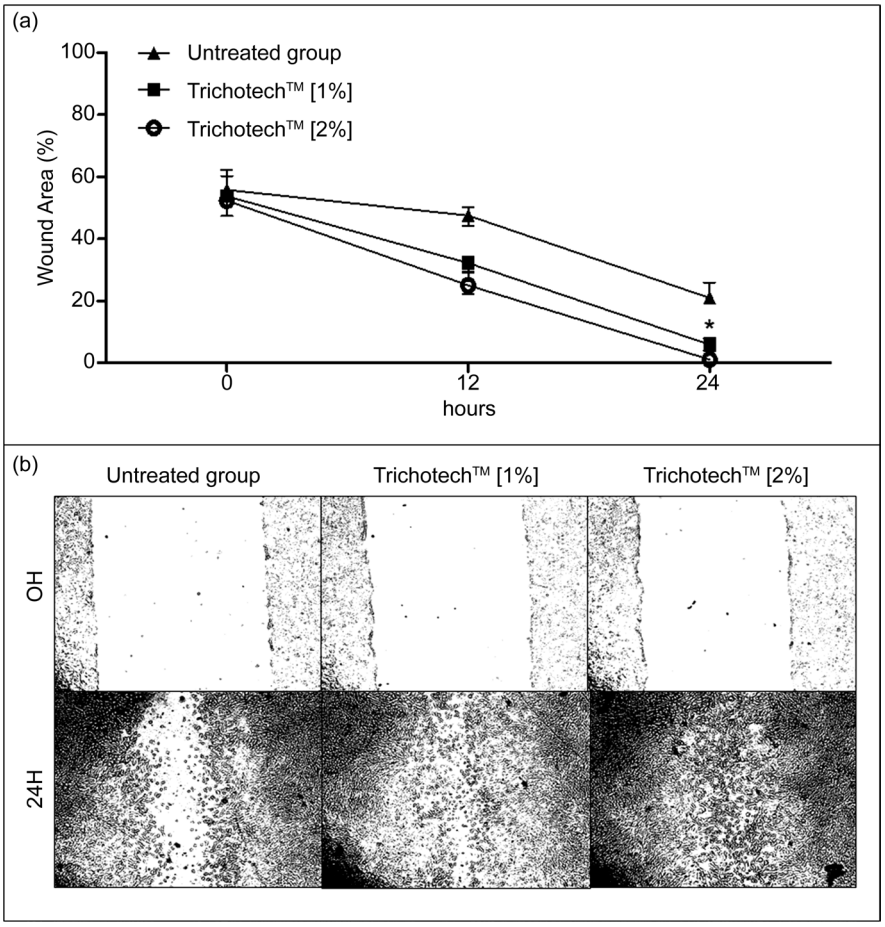

Figure 3. (a) Photographic representation of *in vitro* samples subjected to a simulated wound and exposed for 24 hours to different concentrations of Trichotech™ (1% and 2%). (b) Representative graphic of the percentage of wounded area at 0 h and 24 h after the same treatment. Before starting the tests, cells were deprived of fetal bovine serum. (*) $P < 0.05$—significant in relation to group 0 h treated with Trichotech™, Student's t-test, GraphPad Prism v5.0.

were obtained [21], whereby the proliferative effects of camphor were shown to be mediated by the PI3K/AKT/mTOR and MAP kinase pathways—the key signaling pathways involved in the control of cell proliferation. In this same study, camphor-induced phosphorylation of ERK, but not PI3K and AKT, was also reported. Taken together, this evidence indicates that Trichotech™ induced fibroblast proliferation possibly through upregulation of MAP kinase signaling pathways.

3.5. Real-Time PCR (qPCR)

Several growth factors (e.g., FGF-1, FGF-2, FGF-7 and FGF-10) can promote cell cycle and proliferation and have the potential to rescue hair loss and facilitate hair cell regeneration *in vivo* and *in vitro* [9]. Among these FGF genes, FGF-7 was found to be expressed in the hair follicle. FGF-7 RNA is localized to the dermal papilla during anagen, but expression is down-regulated by the late-anagen VI stage [22]. Besides, no FGF-7 RNA was detected in follicles during

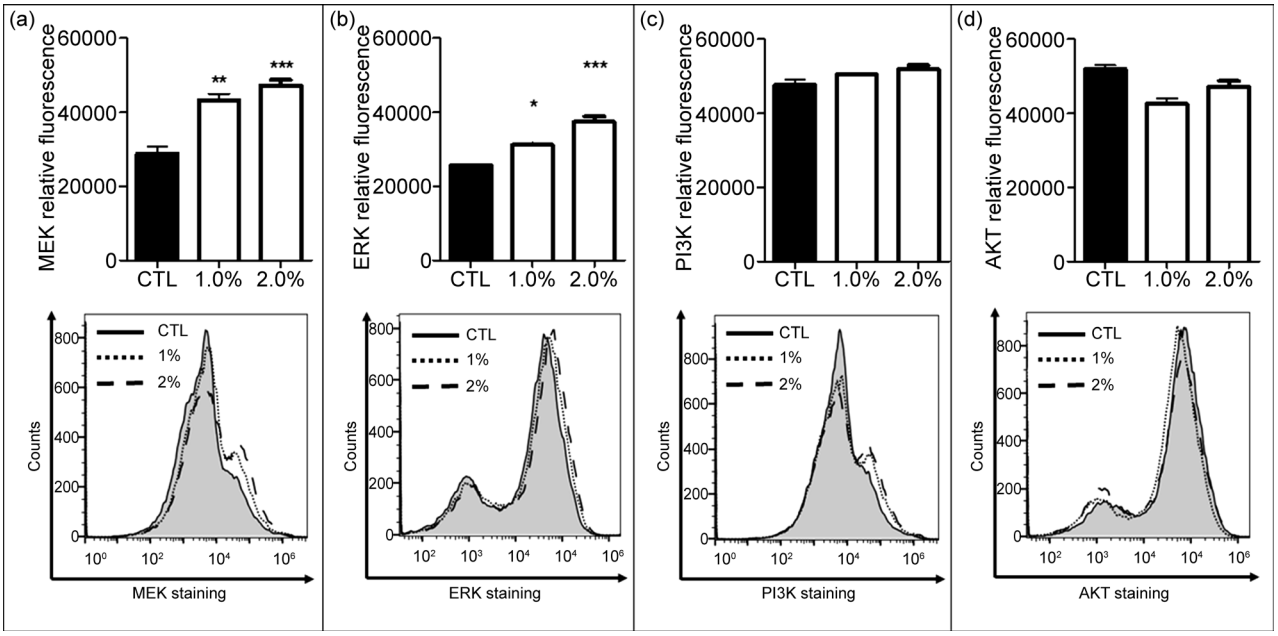

Figure 4. Graphic and corresponding representative histogram of MEK (a), ERK (b), PI3K (c) and AKT (d) phosphorylated proteins signaling after exposure to Trichotech™ [1%] and [2%] for 1 h. Results are expressed by the MFI (median fluorescence intensity) and compared with the untreated group (CTL). (*) $P < 0.05$—significant in relation to CTL group. ANOVA, Tukey, GraphPad Prism v5.0.

catagen or telogen [23]. FGF-10 is found in the dermal papilla fibroblasts and its receptor FGFR2IIIb is found in the neighboring outer root sheath of the keratinocytes [24], suggesting that FGF-10 is a mesenchymally derived stimulator of hair follicle cells, which contribute to the hair-promoting activity. To verify the relative expression of mRNA for fibroblast growth factor-7 (FGF-7) and fibroblast growth factor-10 (FGF-10) genes, a real-time polymerase chain reaction (qPCR) protocol was used. **Figure 5** shows the increase in mRNA expression by fibroblasts after treatment with Trichotech™. In both concentrations tested, Trichotech™ was found to increase the expression of FGF-7 and FGF-10 by several fold, thereby constituting a dermal papilla signal instructing hair germ cells to proliferate and initiate a new hair cycle.

3.6. Sirius Red Collagen Quantification

Sirius red staining of collagen has been used for many years. The present colorimetric plate assay allows for rapid assessment of collagen content [25]. The sirius red assay showed a significant increase in collagen content in treated fibroblasts (**Figure 6**). The magnitude of the increase in collagen between control and treated samples was markedly increased following treatment with 2.0% Trichotech™. Previous reports have demonstrated similar increases in collagen production following exposure to natural compounds, akin to the data obtained in the present work. Jung [26] showed that Camellia japonica oil was capable of inducing type I collagen synthesis in fibroblasts. Studies also observed increased type I collagen content in human dermal fibroblasts following exposure to ginseng and cinnamon preparations, respectively [27] [28].

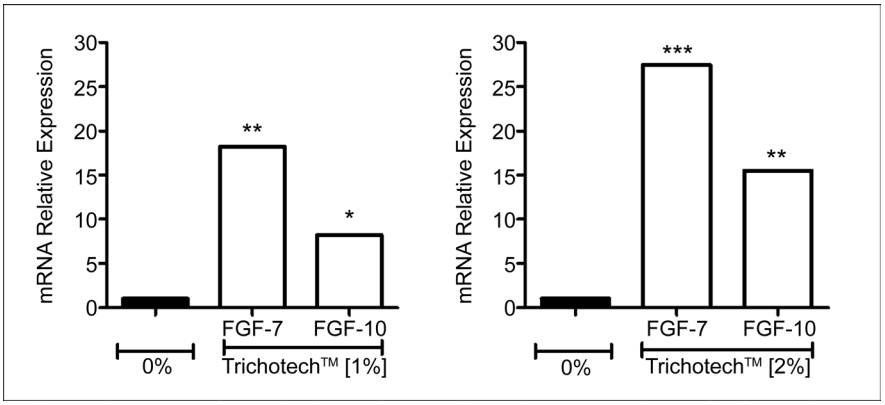

Figure 5. Relative expression levels of mRNA for FGF-7 and FGF-10 in human fibroblasts assessed by quantitative RT-PCR. Bars represent the range of relative expression. *P < 0.05, **P < 0.01, and ***P < 0.0001 compared to internal control GAPDH. Graphs were plotted with GraphPad Prism v5.0.

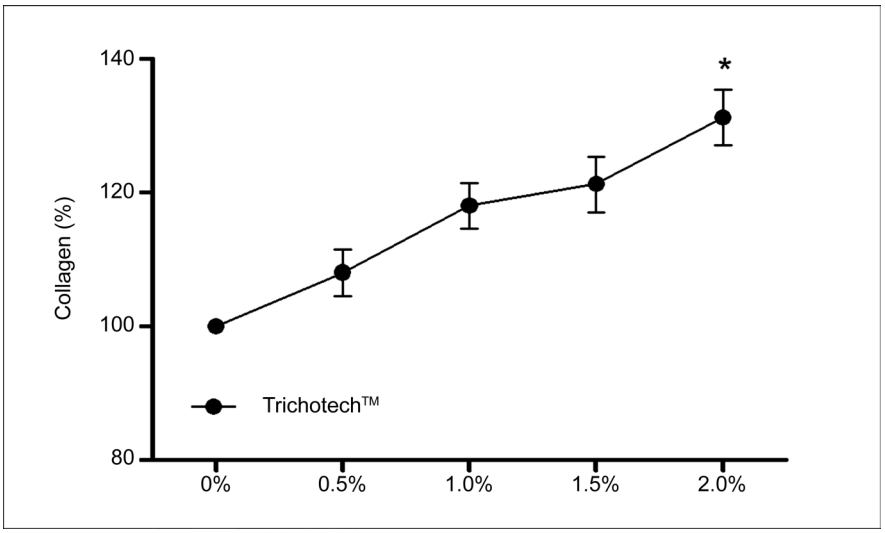

Figure 6. Total collagen content in fibroblasts as measured by incorporation of Sirius Red dye after 24-hour exposure to different concentrations of Trichotech™. Before starting the tests, cells were deprived of fetal bovine serum. The exposure did not cause a significant increase in relation to the control group (untreated cells). (*) P < 0.05—significant in relation to control. Student's t-test. GraphPad Prism v5.0.

4. Conclusion

Fibroblasts are found in the dermal papilla of hair follicles, and as such are heavily implicated in hair growth regulation. Taken together, our data show a stimulating effect of Trichotech™ on cultured fibroblasts. Indeed, a pronounced increase in cell growth was observed after exposure to Trichotech™ at concentrations of 0.5% - 2.0%. Our results suggest that Trichotech™ induced fibroblast proliferation by activating ERK signaling pathways. In addition, FGF-7 and FGF-10 mRNA levels were shown to be increased compared with untreated controls. Further investigation concerning Trichotech™ could be helpful towards the development of new bioactive phytocomplexes for dermatological and trichological use.

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