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The Effects of *Helichrysum italicum* Extract on the Extracellular Matrix of the Skin

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Abstract
Objective. An in-vitro study was performed to investigate the molecular basis of the wound healing and skin protective features of *Helichrysum italicum* (*HI*), a medicinal plant from the Mediterranean basin. **Materials and Methods.** A dermal fibroblast cell line culture was treated with *HI* hydro-alcoholic extract to detect the gene expression levels of three selected primers: *FGF-2*, *HAS-2* and *MMP-9*. Cell proliferation assay was performed using a XTT reagent. RNA isolations were carried out from both the extract treated study cell group and the control cell group using a TRI reagent. *GAPDH* was used as the reference gene. Gene expressions were determined by real time RT-qPCR. The results were represented as 'Target/*GAPDH* Fold Change'. Statistical evaluation was performed by Student's t test. **Results.** *HI* extract caused statistically significant upregulation of *FGF-2* (P=0.0473) and *HAS-2* (P=0.0335) gene expressions compared to the untreated control cells. The treatment ended with 1.74 and 3.10 fold changes for *FGF-2* and *HAS-2*, respectively. **Conclusion.** In general, it may be considered that *HI* has certain anabolic effects on the extracellular matrix of the skin because of the significant increases it causes in *FGF-2* and *HAS-2*. Therefore, it may have a promising future in anti-aging studies and cosmetic dermatology. The results obtained in this study may also partially explain the molecular basis of the health benefits of *HI* on skin, including improvement in wound healing, and protection against the detrimental effects of ultraviolet exposure.

Key Words: *Helichrysum italicum* ▪ FGF-2 ▪ HAS-2 ▪ MMP-9.

Introduction

Medicinal plants are important sources of novel drug discoveries (1). Plants contain miscellaneous molecules with significant pharmacological actions. The genus *Helichrysum* (family *Asteraceae*) includes more than one thousand species and subspecies, most of which grow in the Mediterranean basin (2). There are numerous reports on the traditional uses of *Helichrysum italicum* (*HI*) or the "everlasting plant" in Northern Mediterranean countries. Data from various ethnopharmacological surveys show that the most frequently reported traditional uses of *HI* are related to respiratory diseases, digestive disorders, wound healing and inflammatory skin conditions. Wound healing and skin protective properties seem to be the best documented therapeutic effects of *HI*, as shown by in vivo studies performed with topical application of its extracts (3). A large variety of extracts of *HI* can be prepared, including the volatile oil, and the resulting products differ in their chemical compositions (4-6). Therefore *HI* bioactivity may show differences, depending on the chemical composition of its different extracts, from which most of the main active compounds have already been isolated (5). Extraction with organic solvents, such as ethanol, methanol and acetone, is most frequently used to attain non-volatile *HI* extracts, whereas hydrodistillation and steam distillation are favored for the isolation of volatile essential oils (7). *HI* extracts and essential oils have a wide variety of chemical classes, among which flavonoids, α-pyrones, phenolic acids, acetophenones, tremetones, pholoroglucinol derivatives monoterpenes, sesquiterpenes, and triterpenes dominate (3, 7). Flavonoids, acetophenones, and pholoroglucinol derivatives have been shown to have inhibitory activity in different pathways of arachidonic acid metabolism and other pro-inflammatory mediators (3). It was reported that the flavonoid fraction may protect skin from ultraviolet (UV)-induced damage by a combination of UV-absorbing, DNA-protective, anti-oxidant, and anti-inflammatory properties (8). Arzanol, a heterodimeric phloroglucinol identified as the major anti-oxidant, anti-inflammatory and anti-viral constituent of *HI* (4, 9), potently inhibits the biosynthesis of pro-inflammatory lipid mediators, such as prostaglandin E_2 (PGE_2), both in vitro and in vivo. It also showed potent antibacterial action against multidrug-resistant *Staphylococcus aureus* isolates (9). Phytochemical investigations of *HI* essential oil revealed some sesquiterpenes contents, such as γ-curcumene and β-selinene, as well as monoterpenes, such as α-pinene and neryl acetate. These terpenes, as the most characteristic constituents, might be effective as potential wound healing agents (10). Neryl acetate was also demonstrated to strengthen the skin barrier function by increasing lipid and ceramide content in the stratum corneum, through increasing the expressions of ceramide synthesis-related enzymes (11). These observations validate the topical use of *HI* extracts to prevent wound infections in the traditional medicine of the Mediterranean area (12).

In this study, we investigated the effects of *HI* extract on a fibroblast cell line with selected factors which are all highly effective in the metabolism of the cutaneous extracellular matrix (ECM). These were *Fibroblast Growth Factor-2* (*FGF-2*), a potent mitogen for the cells of mesenchimal and neuroectodermal origin (13); *Hyaluronan Synthase-2* (*HAS-2*), the major enzyme synthesizing hyaluronic acid (HA) (14); and *Matrix Metalloproteinase-9* (*MMP-9*), an enzyme with essential roles in basement membrane remodeling through its proteolitic activity (15).

Methods

Plant Material and Preparation of the Extract

Only the flower part of the plant was used. Five grams of dried yellow flowers were extracted with 500 mL of distilled water-ethanol mixture (70:30 v/v) using soxhlet apparatus for two cycles. The extract was filtered through 0.45 μm filter paper and kept in a refrigerator at between 4-8°C until further analysis. The extract was evaporated in a rotavapor until 5% dissolved solids content remained. The 5 brix extract was used for the cell culture experiments. The solvent alcohol was eliminated during evaporation under vacuum. The final solution was a concentrated aqueous extract, and the dissolved botanical content of the extract was 50 mg/mL.

Cell Culture

Human skin fibroblast cells (HSF 1184) were cultured in Dulbecco's Modified Eagles Medium with high glucose, supplemented with 15% heat-inactivated fetal bovine serum and 1% gentamicin. The cells were maintained at 37°C in a humidified atmosphere at 5% CO_2 in a Newbrunswick incubator. All supplements and media were purchased from Sigma Aldrich.

Cell Proliferation Assay and Cytotoxicity Analysis

The cellular toxicity of *HI* extract was investigated using 2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-S-|(phenylamino)carbonyl|-2//-tetrazolium hydroxide (XTT) cell proliferation assay (Roche Diagnostics) according to scientific principles (16) and manufacturers' instructions. The cells were seeded into 96-well plates (10^4cells/well) and incubated for 24 h at 37°C, in a humidified atmosphere at 5% CO_2. On the second day, new medium was added, after aspiration of the previous one, subjected to different concentrations (100%, 10%, 5%, 0%) of the extract and incubated in the same conditions for 72 h. XTT reagent was added to the plates after the incubation period to obtain

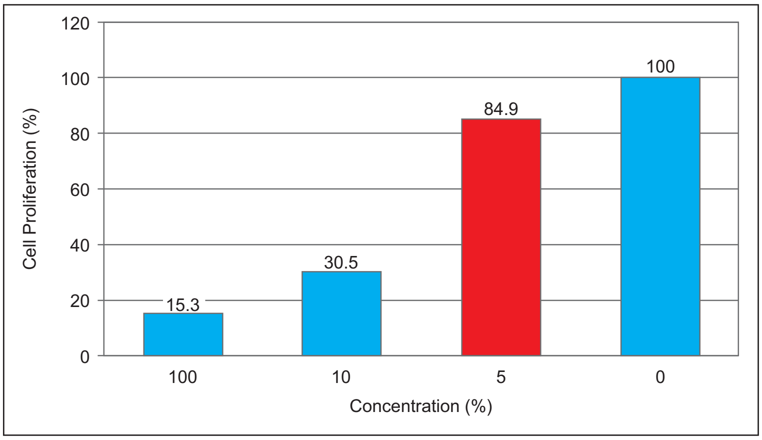

Figure 1. Cytotoxicity results of *HI* extract. The red bar represents the concentration used for incubation.

a concentration of 0.3 mg/mL. Then the cells were incubated at 37°C for 4 h in order to reduce the XTT reagent to an orange formazan compound. The optical density of the soluble formazan compound was measured at 450 nm, with 650 nm reference level by microplate reader (Bio-Rad). On the basis of the cell proliferation ratios of the treated cells with respect to the control cells, the cytotoxicity levels of the extract were determined. Higher concentrations were found to be cytotoxic for fibroblast cells. For the subsequent analysis, the possible highest concentration was determined as 5%, having optimum cell viability of approximately 80%, and the fibroblast cells were incubated with a 5% concentration of extract solution before total RNA isolation (Figure 1).

Reverse Transcription

Total RNA was extracted from the cells treated with *HI* extract solution and from untreated cells, using the TRI reagent (Sigma Aldrich) according to the scientific principles (17, 18) and the manufacturer's instructions. The concentration and purity of the isolated RNA samples were determined by measuring optical densities at 260 nm and 280 nm using BioSpec-nano. A Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics) was used for cDNA synthesis. 500 ng total RNA and 10 mM gene specific primers of *FGF-2*, *HAS-2*, *MMP-9* as study material, and *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) as the reference gene (Integrated DNA Technologies) were added to RNAse free test tubes and the final volume was reached at 13 µL for each, by adding distilled water. After incubation for 10 min at 65°C in a Thermal Cycler, the tubes were transferred over ice. Later they were incubated for 30 min at 55°C and 5 min at 85°C in a Thermal Cycler, after adding 4 µL of Reverse Transcription Buffer (5X), 2 µL of dNTP mix (10 mM), 0.5 µL of Protector RNAse Inhibitor and 0.5 µL of Reverse Transcriptase. The primer sequences are given in Table 1.

Gene Expression Analysis

A Fast Start DNA Green Master Kit (Roche Diagnostics) was used for the real-time reverse transcription quantitative polymerase chain reactions (RT-qPCR). The analysis was performed according to the scientific principles (17, 18) and the manufacturers' instructions. Briefly, the total volume of reaction mix was 20 µL, containing 10 µL Master Mix, 10 mM of reverse and forward

Table 1. Primers (5'–3') of the Genes Studied

Primers	Forward primer	Reverse primer
FGF-2	CCTCAAGGTTCTCAAGGCCG	AGCACGTATATTCCCCAGCG
HAS-2	GCCTGGGCTATGCAACAAAA	GTAGGACTTGCTCCAACGGG
MMP-9	GTACTCGACCTGTACCG	AGAAGCCCCACTTCTTGTCG
GAPDH	ATGGGTGTGAACCATGAGAA	GTGCTAAGCAGTTGGTGGTG

FGF-2=Fibroblast growth factor-2; HAS-2=Hyaluronan synthase-2; MMP-9=Matrix metalloproteinase-9; GAPDH=Glyceraldehyde-3-phosphate dehydrogenase.

primers, 25 ng template cDNA and the appropriate amount of RNAse free distilled water. All samples were run as triplicates in each run, including a non-template control and four standards (1:1, 1:10, 1:100, 1:1000). The real-time RT-qPCR parameters were determined separately for each target according to the melting and annealing temperatures of the primers. Each parameter included a pre-incubation step for 10 min at 95°C, followed by 45 cycles of three amplification and melting steps. Melting curve analysis was performed to verify specificity. Absolute quantification analysis was performed using a Light Cycler 96 (Roche Diagnostics). For quantitation of real-time RT-qPCR results, the $\Delta\Delta C_t$ method was used. The gene expression results were represented as 'Target/*GAPDH* Fold Change'.

Statistical Analysis

All data were representative of the three experiments and expressed as mean ± standard deviation, together with 95% confidence interval (CI). Statistical evaluation was performed by Student's t test (Graph Pad Prism 6), and statistical significance was defined as $P<0.05$.

Results

HI hydro-alcoholic extract caused statistically significant upregulation in human skin fibroblast cells for *FGF-2* (P=0.0473) and *HAS-2* (P=0.0335) gene expressions. The treatment resulted in 1.74±0.26 (95% CI: 1.74±0.21) and 3.10±0.76 (95% CI: 3.10±0.61) fold changes for *FGF-2* and *HAS-2*, respectively. Also, as a positive outcome, the treatment resulted in a 0.72±0.19 (95% CI: 0.72±0.16) fold change for *MMP-9* gene expression, however, the result was not statistically significant. The fold changes and the P values of the gene expression analyses are given in Figure 2.

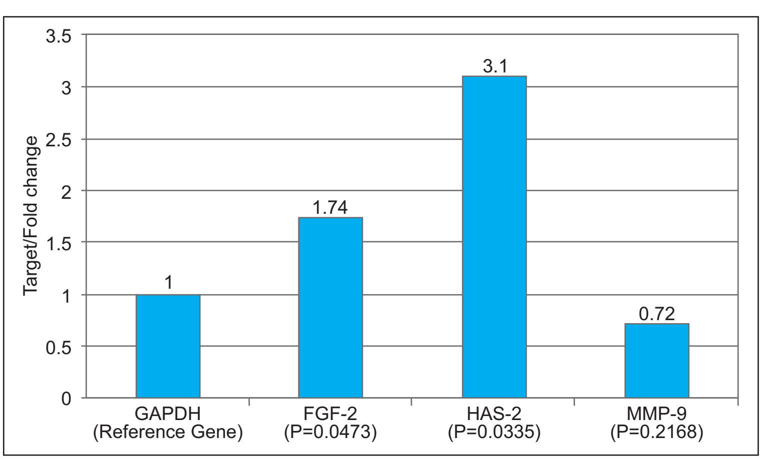

Figure 2. Gene expression levels and P values of *FGF-2*, *HAS-2* and *MMP-9* after treatment with *HI* extract, compared to untreated control cells.

Discussion

In our results, there were statistically significant increases in *FGF-2*, and *HAS-2*, and some decrease was recorded in *MMP-9* which was not statistically significant. Long term exposure to environmental or internal disturbances cause tissue damage through the formation of reactive oxygen species and the decline of cell functions. These inflammatory reactions increase the synthesis of dermal enzymes which brings on the degradation of ECM (19). The *FGFs 1* and *2*, also known as acidic and basic *FGF*, respectively, are produced by inflammatory cells, vascular endothelial cells, fibroblasts and keratinocytes. They are expressed upon dermal injury and have important functions in re-epithelization, angiogenesis, and granulation tissue formation (20). *FGF-2* increases the synthesis of matrix macromolecules, and notably that of HA, by stimulating the expression of *HAS* genes (21). HA is an essential component of the skin, responsible for captivating water and giving the dermis its volume (22). The concentration of HA in various tissues is in correlation with the transcription of *HAS* genes, especially with *HAS-2* (14). It was demonstrated that *HAS-2* protects skin fibroblasts against apoptosis, induced by environmental stress, mainly UV-B (23). The synthesis of HA, regulated by *HAS-2*, conducts keratinocyte migration, which is crucial for the reconstruction of squamous epithelia after wounding

(24). *MMPs* are secreted by keratinocytes and dermal fibroblasts in reaction to various stimuli, such as oxidative stress, UV and cytokines (25). *MMP-9* is thought to have critical functions in the remodeling of the basement membrane zone because several ECM proteins in this region have been determined as substrates of this proteinase (15).

Considering the significant increases in *FGF-2* and *HAS-2* gene expressions in our study, it may be suggested that *HI* has some anabolic effects on the ECM of the skin, mainly due to the angiogenesis inducing and granulation tissue enhancing effects of *FGF-2* (20), and the fibroblast protecting activities of *HAS-2* against UV-B mediated stress (23). The results obtained in this study may also partially explain the molecular basis of the health benefits of *HI* on skin, including improvement in wound healing, and protection against the detrimental effects of UV exposure. It is quite possible that these results are largely related to the plant's strong anti-inflammatory and anti-oxidant activities (3, 5, 8, 9, 12). Suppression of the degradation and increasing the synthesis of the ECM components of the skin are also the well known targets of anti-aging studies. Therefore, regarding the anabolic effects of *HI* in dermal ECM, it may have a promising future in cosmetic dermatology.

Limitations of the Study

Although the strong anti-oxidant and anti-inflammatory activities of *HI*, related to its phenolic and flavonoid constituents, have been revealed in recent studies performed by other researchers, the lack of phytochemical analyses in the particular plant of our study, prevents us from establishing a solid connection between the current results and the chemical features of the plant.

Conclusion

In general we may consider that *HI* has some anabolic effecst on the ECM of the skin because of the significant increases it induced in *FGF-2* and *HAS-2*. The results obtained by this study may also partially explain the molecular basis of the health benefits of *HI* on skin, including improvement in wound healing, and protection against the detrimental effects of UV exposure.

What Is Already Known on This Topic:

There are numerous reports on the traditional uses of HI in the Northern Mediterranean countries. Although data from various surveys show that the most frequently reported uses of HI are related to respiratory diseases, wound healing, digestive disorders and inflammatory skin conditions, its wound healing and skin protective properties seem to be the best documented therapeutic effects of this plant. HI's bioactivity depends on the chemical composition of its different extracts, from which most of the main active compounds have been isolated. These compounds are mainly flavonoids, acetophenones, pholoroglucinol derivatives and terpenes, which have been demonstrated to have anti-inflammatory, anti-oxidant, anti-microbial and wound healing features. Despite these scientific data, the molecular basis of the suggested activities is still lacking. Therefore, we performed an in vitro study to reveal the activities of HI on skin fibroblast cells, to see whether it has an effect on FGF-2, HAS-2 and MMP-9, the three functional proteins of skin.

What This Study Adds:

Considering the significant increases in FGF-2 and HAS-2 gene expressions in our study, it may be suggested that HI has some anabolic effects on the extracellular matrix of the skin, mainly due to the angiogenesis inducing and granulation tissue enhancing effects of FGF-2, and the fibroblast protecting activities of HAS-2 against UV-B mediated stress. The results obtained in this study may also partially explain the molecular basis of the health benefits of HI on skin, including improvement in wound healing, and protection against the detrimental effects of UV exposure.

Authors' Contributions: Conception and design: EP and MT; Acquisition, analysis and interpretation of data: EP and MT; Drafting the article: EP and MT; Revising it critically for important intellectual content: EP and MT; Approved final version of the manuscript: EP and MT.

Conflict of Interest: The authors declare that they have no conflict of interest.

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