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Original research article

The concentration-dependent effect of anethole on collagen, MMP-2 and GAG in human skin fibroblast cultures

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A R T I C L E I N F O

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A B S T R A C T

Purpose: In aging skin and some skin disorders, components of skin extracellular matrix (ECM) are disturbed and therefore research to find skin drugs is important. Evaluation of anethole impact on collagen, GAGs and MMP-2 in human skin fibroblasts was the aim of this study.
Materials and methods: For collagen assay the Sircol dye, 5-[^{3}H]proline and real time-PCR were used. MMP-2 activity was detected by zymography. GAG concentration was determined using 1,9-dimethylmethylene blue (DMMB). Cell viability was assayed with MTT.
Results: In cells treated with 1 and 10 μM anethole, a significant increase in collagen synthesis was demonstrated. In contrast, collagen synthesis was significantly decreased in cells exposed to 100 μM anethole. Similar alterations were found in collagen type I expression. The concentration of collagen secreted into the medium was higher only in cells exposed to 1 μM anethole, while it was lower under the influence of higher compound concentrations. It may be due to the lack of pro-MMP-2 activation at 1 μM and a significant increase in the level of MMP-2 at 10 and 100 μM anethole. GAG concentration was reduced under the influence of 100 μM anethole, whereas anethole at lower concentrations revealed the ability to prevent H_2O_2-induced GAG increase. No significant cytotoxicity of anethole to fibroblasts was noted.
Conclusions: Our findings demonstrate the concentration-dependent action of anethole on the crucial components of ECM in cultured skin fibroblasts, which may be somewhat beneficial and may possibly be developed towards a therapeutic use in some skin disorders.

1. Introduction

Essential oils are volatile secondary metabolites extracted from different parts of the plants such as flowers, seeds, leaves, fruits and roots, having wide application in the pharmaceutical, food, cosmetic and perfume industries. They exhibit multiple biological activities, i.e. antibacterial, antifungal, insecticidal, antioxidant, anti-inflammatory, anti-carcinogenic and their medicinal potential depend on their chemical composition [1–4]. Therefore, to understand the mechanism of pharmacological action of essential oils, the examination of their chemical components is very important.

Many research studies provided evidence about the beneficial properties and potential therapeutic applications of (*E*)-Anethole [1-methoxy-4-(1-propenyl)benzene] (Fig. 1). It is a volatile simple compound which was isolated as a potential insecticide and sweetening substance from the fruits of *llicium verum Hook. f. known as the* Chinese star anise [5,6]. It is also a major component of anise (*Pimpinella anisum* L.), sweet anise (*Foeniculum vulgare* Mill.) and *Croton zehntneri* Pax & K. Hoff, essential oils, where anethole content is higher than 80% [7–9]. In contrast, Japanese star anise (*Illicium anisatum* L.) contains less than 3.0% of anethole. In lower concentration, it was also found in the essential oils of lemon balm, cilantro, and basil. These oils are known for their use in traditional medicine as expectorant and carminative drugs as well as for the treatment of various conditions, particularly skin inflammation, stomach aches and rheumatic pain [9]. Anethole, like many other components of essential oils, exhibits antibacterial [2,7,10], antifungal [11], antihypernociceptive [12], antioxidant [2,10,13–15], anti-inflammatory [14–18], and anti-carcinogenic action [15,19]. In addition to the anticancer potential of anethole, it may be also effective in the prevention and treatment of diseases related to nervous system

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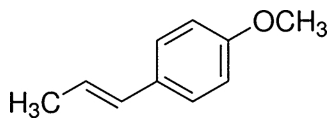

Fig. 1. Chemical structure of anethole.

[20], skeletal muscles [21], cardiovascular diseases [22], diabetes [23], gastrointestinal problems [14,24] and wound healing [25].

In our previous study, we have shown the ability of anethole to prevent changes in the viability of human skin fibroblasts and collagen production caused by H_2O_2 [26]. In the present study we decided to evaluate, in cultured human dermal fibroblasts, the direct effect of anethole on collagen, matrix metalloproteinase (MMP-2) and glycosaminoglycans (GAGs), which are important subjects in the skin anti-aging and anti-fibrotic strategies.

2. Materials and methods

2.1. Plant material

Investigated compound (*E*)-anethole was isolated from a commercial anise fruit essential oil (Pollena-Aroma, Warsaw, Poland) and identified with GC and GC–MS methods as previously described [26]. The purity of compound was established at 99%.

2.2. Fibroblast culture

Primary Dermal Fibroblast: Normal, Human, Adult ATCC® PCS-201-012™ was purchased from ATCC (Manassas, VA, USA). The cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (HyClone Laboratories, Logan, UT, USA), 2 mM glutamine, 50 U/mL penicillin, and 50 μg/mL streptomycin at 37 °C in a 5% CO_2 incubator. For experiments, cells were plated at a density of 1×10^6 per well in 6-well culture plates (Costar).

Anethole dissolved in dimethyl sulfoxide (DMSO) was added to the medium to a final concentration of 1, 10 and 100 μM and incubated for 24 h. The same concentration of DMSO solution (0.01%) was used as a control in order to rule out the possible effect of DMSO on fibroblasts.

2.3. Cell viability

The assay was performed using [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT). Confluent cells cultured with the tested compound for 24 h at 37 °C were washed with phosphate-buffered saline (PBS) and then incubated for 4 h in 1 mL MTT solution (0.5 mg/ml). The medium was removed and 1 mL of 0.1 M HCl in absolute isopropanol was added to the cells. Absorbance of converted dye in living cells was measured at a wavelength of 570 nm. Viability of cells incubated in the presence of anethole was calculated as a percent of the control cells.

2.4. Collagen biosynthesis

Confluent cells were incubated with different concentrations of anethole (1, 10 and 100 μM) and 5 μCi L-[5-^{3}H]proline (28 Ci/mmol) for 24 h. Incorporation of radioactive precursor into collagen was determined by digesting the proteins with purified *Clostridium histolyticum* collagenase according to the method of Peterkofsky and Diegelmann [27]. Cells were washed three times with sterile 10 mM PBS, pH 7.4, and cell membranes were disrupted using a sonicator (Sonics Vibra cell, USA). Aliquots of the cell homogenate were removed for protein measurement using the BCA™ Protein Assay Kit (Pierce). The remaining homogenate was used for analysis of collagen synthesis.

2.5. Sircol collagen assay

Confluent cells were pre-incubated for 2 h in the serum-free medium and then anethole at concentrations of 1, 10 and 100 μM was added. Incubation was prolonged to 24 h. Concentration of total soluble collagen secreted into the medium was measured using the Sircol collagen assay kit (Biocolor Assays, Ireland). Briefly, 1 mL of Sircol dye reagent was added to the 100 μL of concentrated medium and mixed for 30 min on an orbital shaker. The samples were then centrifuged at $9.3 \times g$ for 10 min and the dye bound to the collagen pellet was dissolved in 1 mL of 0.5 M NaOH solution. The absorbance of the samples was measured at 555 nm using a microplate reader (Infinite M200, Tecan, Austria). A calibration standard of type I collagen, provided with the assay, was used to obtain the standard curve.

2.6. Gelatinase zymography

Equal amounts (10 μg) of the serum-free medium proteins were electrophoresed under non-reducing conditions in 10% polyacrylamide gel impregnated with 1 mg/mL gelatin from porcine skin, Type A (Sigma-Aldrich Corp., USA) as a substrate. Following electrophoresis, the gels were washed twice for 15 min with 2% Triton X-100 to remove SDS and then incubated overnight at 37 °C in the reaction buffer (50 mM Tris − HCl, pH 8.0, 5 mM $CaCl_2$). The gels were stained with 0.5% Coomassie Brilliant Blue R-250 in 50% methanol and 10% glacial acetic acid and then destained. Clear bands on the blue background, representing areas of substrate-degrading enzymes, were quantified using an imaging densitometer (G:BOX, Syngene). Protein molecular weight standard was run concurrently to determine molecular weight of gelatinase.

2.7. Real-time PCR

Extraction of total RNA was performed using the EXTRACTME Total RNA Kit (DNA Gdansk, Poland) according to the manufacturer's protocol. RNA concentration and quality were determined using a NanoDrop 2000 spectrophotometer (Thermo Scientific, USA). Total RNA (1 μg) in the total volume of 20 μL was reverse transcribed using SensiFAST cDNA Synthesis Kit (Bioline). Real-time PCR was carried out, using 2 μL of the 3-times diluted cDNA product, 400 nM each of the primer and the SensiFAST™ SYBR Kit (Bioline), in CFX96 Real-time system (Bio-Rad). Primer sequences (forward and reverse) used to amplify cDNA of type I collagen and GAPDH genes were as follows: *COL1A1* forward, 5′-TAC AGC GTC ACT GTC GAT GGC-3′ and *COL1A1* reverse, 5′-TCA ATC ACT GTC TTG CCC CAG-3′; *GAPDH* forward, 5′-GTG AAC CAT GAG AAG TAT GAC AA-3′ and *GAPDH* reverse, 5′-CAT GAG TCC TTC CAC GAT AC-3′. As cycling parameters were: 95 °C for 1 min to activate the DNA polymerase, 40 cycles of denaturation for 10 s at 95 °C, annealing for 15 s at 60 °C, and extension for 20 s at 72 °C. The reaction was then subjected to a melting protocol from 55 °C to 95 °C with a 0.2 °C increment and 1 s holding at each increment to check the specificity of the amplified products. Single product formation was confirmed by melting point analysis and agarose gel electrophoresis. Samples were run in triplicate and the ΔΔCT method was applied for a statistical analysis of the CT-values. The relative gene expression levels were standardized with those measured in the non-treated with anethole control.

2.8. GAGs content assay

After 24 h incubation of fibroblasts with anethole, the medium was removed and digested overnight with 50 μg/ml proteinase K in 100 mM K_2HPO_4 pH 8.0 at 56 °C. Proteinase K was then inactivated by heating for 10 min at 90 °C. Next, 1 mL of 1,9-dimethylmethylene blue (DMMB) solution was added to the samples and vigorously vortexed for 30 min to promote complexation of GAGs with DMMB [28,29]. The GAG/

DMMB complex was precipitated from solution by centrifugation (12 000 × g) for 10 min. To dissolve the complex, 1 ml of the decomplexation solution was added and the mixture was shaken for 30 min [29]. After this time the absorbance of the samples was measured at 656 nm. GAG content was determined by comparison with a calibration curve of chondroitin sulfate (CS) solutions used as a standard and normalized to the total protein. In experiment on the protective role of anethole against the action of H_2O_2, cells were pre-treated with anethole for 1 h prior to H_2O_2 (0.3 mM) adding and incubated for 24 h.

2.9. Statistical analysis

In all experiments, the mean values for three assays ± SD were calculated. The results were subjected to statistical analysis using the one-way analysis of variance (ANOVA) followed by the Duncan's multiple range post hock test. Differences were recognized as statistically significant at $p < 0.05$.

2.10. Ethical issues

The approval of the Bioethical Committee was not required because the study was carried out on a commercially available fibroblast skin cell line.

3. Results

3.1. Effect of anethole on cell viability

To determine the effect of anethole on viability of human skin fibroblasts, a MTT assay was performed. As shown in Fig. 2, anethole at the concentration of 1 and 10 μM decreased the cells viability by about 10% and at 100 μM by 14%, but these differences were not statistically significant as compared to the control.

3.2. Effect of anethole on collagen synthesis and type I collagen expression

Collagen synthesis in the cells was measured by 5-[^{3}H]proline incorporation into proteins susceptible to the action of bacterial collagenase. We found that anethole, used at lower concentration of 1 and 10 μM, exerted the stimulatory effect on collagen synthesis, increasing it by 31% and 20%, respectively (Fig. 3A). In contrast, exposure of skin fibroblasts to 100 μM anethole, resulted in decreased incorporation of 5-[^{3}H]proline by 32%. To examine whether the stimulatory or inhibitory effect of anethole on collagen occurs at the mRNA level, real-time PCR for the main skin collagen type I was performed (Fig. 3B). We found the positive correlation between the alterations in total collagen synthesis and type I collagen expression suggesting that anethole may affect collagen at the transcription level.

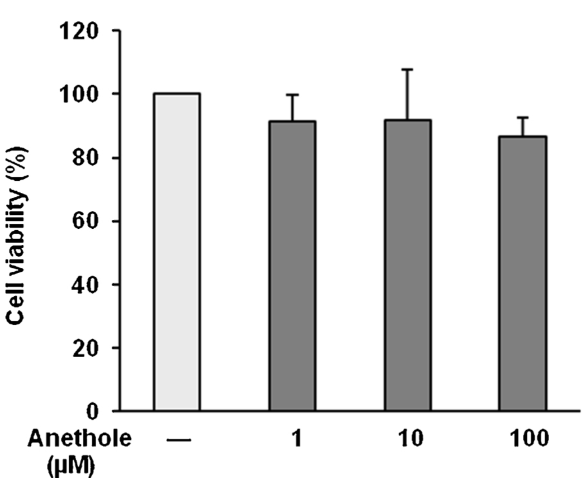

Fig. 2. Effect of anethole on viability of human skin fibroblasts. The cells were subjected to various concentrations of anethole (1, 10 and 100 μM) for 24 h. Cell viability was assessed by MTT test and presented as a percentage of the corresponding control. Values represent the mean (% of control) ± SD of three experiments.

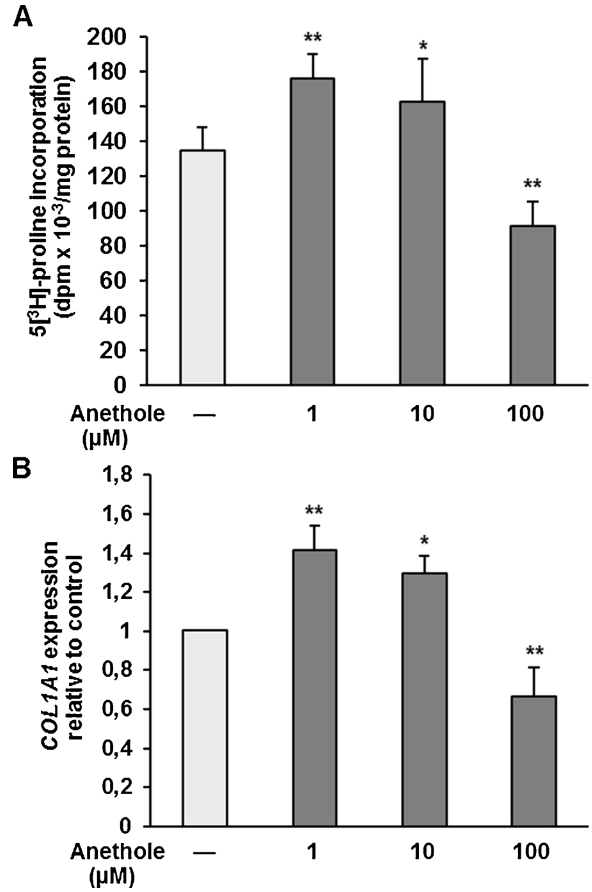

Fig. 3. The effect of anethole on collagen biosynthesis **(A)** and collagen type I gene (*COL1A1*) expression **(B)** in dermal fibroblasts. The cells were subjected to various concentrations of the compound (1, 10 and 100 μM) for 24 h. Values represent the mean ± SD of three experiments. The asterisks (*) indicate statistically significant differences compared to control non-treated with anethole (*P < 0.05, **P < 0.005 vs. control).

3.3. The extracellular collagen concentration and MMP-2 activity

Collagen is the main protein of the skin extracellular matrix (ECM) and therefore we estimated its concentration in the medium using the Sircol collagen assay kit. A statistically significant increase (25%) in collagen concentration was observed in the medium exposed to 1 μM anethole, whereas 10 and 100 μM anethole treatment resulted in a decrease in the collagen level by 15 and 75.3%, respectively, as compared to the control (Fig. 4A).

Collagen like other components of ECM are degraded by MMPs which are zinc-dependent neutral endopeptidases. Using zymography, two forms of gelatinase-A (latent pro-MMP-2 and MMP-2) were detected in both the control (non-treated) and anethole treated fibroblasts (Fig. 4B). In the control medium the main form was pro-MMP-2 whereas the active form accounted for about 14% of total enzymatic activity. Treatment of cells with anethole changed the ratio of these two forms in a concentration-dependent manner. At 1 μM anethole, suppression of MMP-2 activity was found. In contrast, 10 and 100 μM anethole caused a clear activation of pro-MMP-2, however, the greatest enzymatic activity was demonstrated in the medium exposed to 100 μM

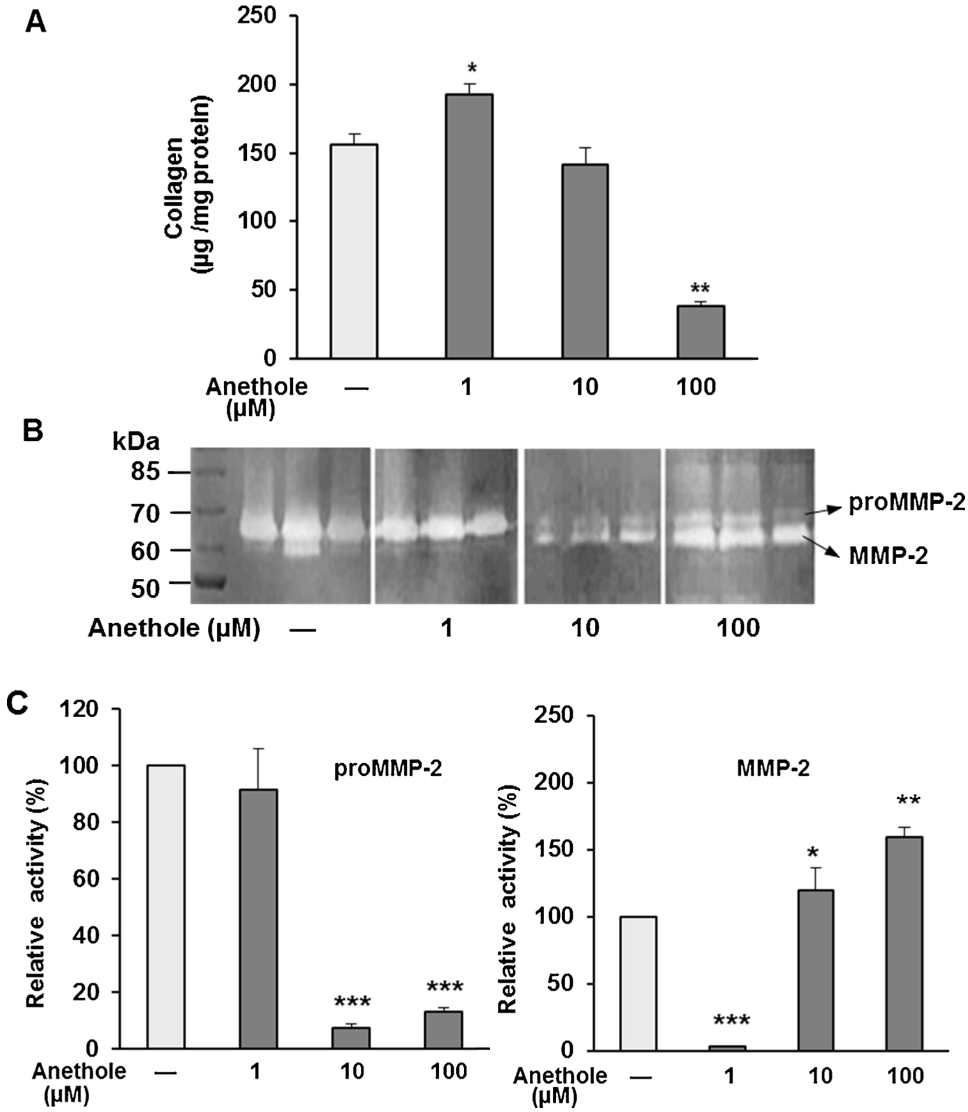

Fig. 4. The effect of anethole (1, 10 and 100 µM) on total collagen, assayed with the Sircol dye **(A)**, and on MMP-2 activity, assayed by zymography **(B)**, in the medium. Values of densitometry represent the mean (% of control) ± SD of three experiments **(C)**. The asterisks (*) indicate statistically significant differences compared to control non-treated with anethole (*P < 0.05, **P < 0.005, ***P < 0.0005 vs. control).

anethole (Fig. 4B, C).

3.4. *Effect of anethole on GAGs*

GAG quantification is based on their ability to bind the cationic dye DMMB. In this assay we used modified method of Barbosa et al. [29] which has improved specificity, reproducibility and sensitivity as compared to the commonly used method of Farndale et al. [28]. As shown in Fig. 5A, anethole exerted a significant inhibitory effect on GAGs in concentration of 100 µM. Because in the previous study we have found the protective effect of anethole on collagen and MMP-2 against the destructive action of H_2O_2 [26], we decided to check if the compound protects GAGs as well. In fact, a significant H_2O_2-induced increase in GAG concentration (about 60%) was normalized in fibroblasts pre-incubated with 1 µM anethole or 50 µM ascorbic acid (used as a positive control) (Fig. 5B). Anethole at 10 µM also suppressed the increase in GAG content, but to a lesser extent than at 1 µM (22% in relation to H_2O_2 treated cells). However, a significant decrease in GAG content was found in fibroblasts pre-treated with 100 µM anethole in relation to both treated and not treated with H_2O_2 cells.

4. Discussion

Collagen, which is the most abundant component of the skin ECM, plays a significant role in maintaining tissue architecture and integrity [30,31]. It is a major protein responsible for the strength, firmness and flexibility of the skin. Furthermore, collagen is not only a structural protein but it also plays an important role in growth, differentiation, and migration of cells as well as angiogenesis, inflammatory response, and interactions between the cells and ECM, mediated by the integrins [31]. Therefore, changes in the structure or quantity of collagen may affect cell signaling and subsequently cellular adhesion, differentiation, growth and other functions of cells.

With age and under the influence of UV, the content of dermal collagen is lowered which causes the loss of tensile strength and elasticity of the skin, increases its fragility and impairs wound healing [32–34]. Loss of the total collagen with age is approximately 1% per unit area of the skin surface per year [32]. The exposure of skin to UV causes reduction of the major collagen type I, which comprises 80–90% of total skin collagen, by 59% [33]. Research aimed at the enhancement of collagen production has great importance in the prevention of photo-aging and in the delay of natural aging. On the other hand, abnormal

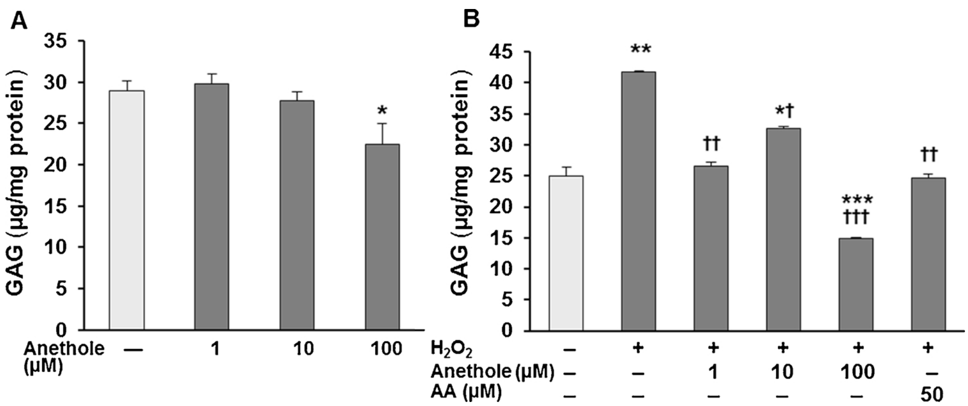

Fig. 5. The effect of anethole (1, 10 and 100 µM) on GAG concentration **(A)** and H_2O_2-induced increase in the GAG concentration **(B)** in skin fibroblasts. Cells were pretreated with anethole (1, 10 and 100 µM) or 50 µM ascorbic acid (AA) for 1 h and then exposed to 300 µM H_2O_2 for 24 h. Values represent the mean (% of control) ± SD of three experiments. The asterisks (*) indicate statistically significant differences compared to control non-treated with anethole ($^{*}P < 0.05$, $^{**}P < 0.005$, $^{***}P < 0.0005$ vs. control); the crosses (†) indicate statistically significant differences compared to H_2O_2-treated cells ($^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.005$, $^{\dagger\dagger\dagger}P < 0.0005$ vs. H_2O_2-treated cells).

biosynthesis of collagen and its local overproduction contributes to the development of certain disorders, e.g. scleroderma, keloids [35,36]. In this case, the agents specifically impairing collagen biosynthesis are required in their treatment.

In prevention of photo-aging and the delay of natural aging the antioxidants and cell regulators are used as components of anti-aging cosmetics [37,38]. The cell regulators such as growth factors have direct effects on collagen production. The antioxidants, e.g. vitamins (C, B_3, E), polyphenols, protect against an increased collagen degradation by reducing the concentration of free radicals in the tissues.

Essential oils and their constituents have a great variety of pharmacological activities and exert beneficial clinical effect on human health [1–4,8,9]. Therefore, they are widely used in cosmetics, sanitary products, dentistry, agriculture, as food preservers and additives, and as natural remedies. Anethole as a major component of anise and fennel fruit essential oil is known for its multidirectional action [5,10,14,19–26].

In the current study we revealed new concentration-dependent activity of the compound that may be somewhat beneficial and may possibly be developed towards a therapeutic use in diseases associated with disturbed metabolism of collagen. Anethole at 1 and 10 µM significantly increased collagen biosynthesis and mRNA expression level of the major skin collagen type I. Because about 85–90% of synthesized collagen is secreted into ECM, we assayed its content in the growth medium. Unexpectedly, higher concentration of collagen was detected only in the medium exposed to the 1 µM anethole. Despite the increased synthesis of collagen under the influence of 10 µM anethole, the concentration of collagen in the medium was lower in relation to the control. Therefore, the compound at the lowest concentration of 1 µM could have beneficial effects in physiological and pathological conditions connected with collagen deficiency. In contrast, anethole at higher concentrations, especially 100 µM, which reduced the synthesis and secretion of collagen into the medium could be considered as skin drug in disorders connected with the excessive collagen production.

The decrease in ECM collagen content can be due to its increased degradation by matrix metalloproteinases, since in the presence of 10 and 100 µM anethole the activation of pro-MMP-2 was revealed. Although MMP-1 is the main enzyme responsible for the fibrillar collagen degradation, it was reported that MMP-2 is also able to cleave the native type I collagen similarly to MMP-1 (to N-terminal 3/4 and C-terminal 1/4 fragment) [39]. Furthermore, it plays a major role in the final degradation of collagen.

GAGs are another important components of the ECM, divided into sulfated (chondroitin sulfate, heparan sulfate and keratan sulfate) and non-sulfated (hyaluronic acid). With the exception of hyaluronic acid (HA) their polysaccharide chains are covalently attached to the protein core forming proteoglycans (PGs) [40,41]. GAG molecules are extremely hydrophilic and assume a highly expanded conformation, creating a hydrogel that is resistant to the high compressive forces. In addition, they interact with the collagen fibers and these interactions play a dominant role in the maintenance of tissue structure and functionality [41]. As in the case of collagen, GAG structure and quantity changes under the influence of various factors, including free radicals.

It has been reported that reactive oxygen species (ROS) decrease collagen production, but increase the synthesis of GAGs in cultured human dermal fibroblasts [42]. In the present study we also found that the exposure of cells to 300 µM H_2O_2 resulted in significant increase (60%) in the GAG content and that anethole at 1 µM completely prevented this alteration. Because results of GAG measurements may be overstated due to the interactions of DMMB with other polyanions present in the cells, we verified the method of Barbosa et al. [29] with some modification regarding pH of DMMB solution and absorbance wavelength [43], but the differences in both GAG assays were very slight (not shown). The beneficial impact of anethole at 1 µM on H_2O_2-induced changes in the synthesis and secretion of collagen and the activity of collagen-degrading enzymes (MMP-2 and MMP-9) was demonstrated in our previous study [26]. Although the mechanism underlying these effects has not been well established, it was shown that this compound and its derivative (anethole ditholethione) can act as ROS scavengers through, among others, the glutathione (the crucial endogenous antioxidant) and glutathione-S-transferase induction [10,13–15,44]. It is also important, that unlike most antioxidants, that exhibit a hydrophilic character, anethole is a small and lipophilic molecule and easily permeates through cell membranes, which increases its therapeutic potential.

5. Conclusions

The results of this study showed beneficial activity of anethole at 1 µM in cultured skin fibroblasts: stimulating impact on collagen, inhibiting pro-MMP-2 activation and preventing GAG against the effect of H_2O_2. The opposite effect of anethole at 100 µM on ECM components suggest its potential in diseases with excessive collagen and/or GAG production. However, further study to clarify the more precise mechanism of anethole action is essential.

Conflict of interest statement

The authors declare no conflict of interests.

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The author contribution

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Data Collection: Edyta Andrulewicz-Botulińska, Iwona Kuźmicz, Joanna Wosek.

Statistical Analysis: Joanna Wosek, Anna Galicka.

Data Interpretation: Edyta Andrulewicz-Botulińska, Jolanta Nazaruk, Anna Galicka.

Manuscript Preparation: Iwona Kuźmicz, Anna Galicka.

Literature Search: Edyta Andrulewicz-Botulińska, Iwona Kuźmicz.

Funds Collection: Iwona Kuźmicz, Anna Galicka

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