

Evaluation of the Stimulating and Protective Effects of Fucoxanthin Against Human Skin Fibroblasts: An *In Vitro* Study

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ABSTRACT

Some plant-derived preparations become increasingly popular in treating unfavorable skin conditions, enhancing beauty and promoting wellness. Active ingredients extracted from marine algae, which are part of the traditional Asian diets and remedies, are actively studied in this respect in recent years. Fucoxanthin (FX), an orange-colored xanthophyll derived from edible brown seaweeds, is among the most intensively studied for use in dermatologic and cosmetic applications. In present study, effects of FX on human skin fibroblasts *in vitro* were investigated. The effect of FX on the cell survival was determined by the tetrazolium-based colorimetric assay (MTT test). The effect of FX on the level of DNA damage in hydrogen peroxide-exposed cells was determined using the Comet assay technique. Data were analyzed by parametric analysis of variance (ANOVA) test and non-parametric Kruskal-Wallis test. According to the MTT test, high concentrations of FX (25, 50 and 100 mcg/mL) were toxic to fibroblasts in a dose-dependent manner, while treatment with low FX concentrations resulted in beneficial (hormetic) effect on cell proliferation. The proliferation rate of cells treated with low FX concentrations (3.13 and 6.25 mcg/mL) was significantly (about 20%) higher than that of the dimethyl sulfoxide (DMSO)-control cells untreated with FX. As was evident from the Comet assay, a significant increase in the level of DNA damage was observed in the H₂O₂-exposed fibroblasts; this unfavorable effect was completely diminished by pre-treatment with 5 mcg/mL of FX. These data are suggestive of a therapeutic potential of FX for dermatological and cosmetological applications.

INTRODUCTION

A growing number of new preparations with a potential for cosmetic dermatology and anti-aging skin care are currently undergoing testing. The use of many of them is controversial, however, because of potential side effects [1, 2]. Therefore, search for more effective and potentially safer treatment alternatives is an urgent task in modern cosmetology. Over the last decade, several plant-derived remedies have consistently demonstrated therapeutic benefits in pre-clinical studies. Many of them become increasingly popular in treating many skin conditions, enhancing beauty and promoting wellness. Active ingredients extracted from marine algae, which are part of the traditional diet and remedies in many Asian countries, are actively studied in this respect in recent years. Due to their unique living environment, seaweeds are rich in bioactive constituents such as vitamins, polysaccharides, phycocyanins, carotenoids, phycobilins, fatty acids and sterols [3]. Algae-based products have been repeatedly

shown to demonstrate broad range of bioactivities including antitumor [4], anti-obesity [5], anti-inflammatory and immunomodulatory [6], and also neuroprotective [7] activities, among others. Growing body of evidence supports the potential of such products in preventing many chronic diseases and promoting overall health [5, 6]. In recent years, algae-derived bioactive components are being increasingly used in dermatology and cosmetology [8].

Since human skin is permanently exposed to potentially hazardous chemical substances and physical agents such as ultraviolet (UV) radiation, the overproduction of reactive oxygen species (ROS) under these exposures is assumed to play an important role in cutaneous pathological conditions such as photoaging, dermatitis, chronic inflammatory processes and skin cancers including melanoma [9]. Therefore, treatment of the skin with antioxidant-containing ingredients is regarded as a useful therapeutic option for preventing oxidative damage and maintaining healthy skin. Fucoxanthin (FX), an orange-colored xanthophyll (natural pigment) derived from edible brown seaweeds, is among the most intensively studied in this context [10]. Although FX can be chemically synthesized, its extraction from macroalgae and microalgae is the most economical and safe source. Among all natural pigments (carotenoids), FX is one of the most abundant; it contributes more than 10% of their estimated total production in nature, especially in the marine environment [11]. Like other carotenoids, FX possesses a variety of beneficial biological activities including radical scavenging and immunomodulation, as well as preventive and therapeutic potential for many disorders such as metabolic syndrome, obesity, heart disease, diabetes, hypertension and cancer [12–14]. Over the last years, protective and therapeutic potential of FX is being actively studied for use in dermatologic and cosmetic applications. Protective effects of FX against UV-caused sunburns were observed [15]. *In vitro* analysis showed that FX can diminish the UV-induced ROS overproduction in human dermal fibroblasts. In a hairless mouse model, topical treatment with FX prevented skin from UV-B induced photoaging [16]. Anti-pigmentary activity of FX along with its influence on skin mRNA expression of melanogenic molecules was revealed in the study by Shimoda et al. [17]. In present study, effects of FX on human skin fibroblasts *in vitro* were investigated.

METHODS

FX solutions preparation

FX was purchased from Sigma Aldrich (USA). Since it is hydrophobic, it was first dissolved in DMSO and then diluted in culture medium to a working concentration (final concentration of DMSO, 0.2 %). Control cells were exposed to the same volume of placebo (0.2 % DMSO) solution. In our preliminary studies, this concentration was shown to be neutral to cell viability (data are not shown). Final concentrations of FX were 0.39, 0.78, 1.56, 3.13, 6.25, 12.5, 25, 50 and 100 mcg/mL.

Skin samples and cultural procedures

A skin biopsy was obtained from three adult-age healthy volunteers with a disposable punch from behind the ear auricle under local infiltrative anesthesia with the 2% lidocaine solution. Isolation and culture of skin fibroblasts was performed according to the method of Rittié [18]. Briefly, the isolated skin fragment (appx. 2 mm²) was immediately transferred to the labeled sterile container with shipment medium (DMEM). Thereafter the biomaterial was transferred under laboratory sterile conditions to Petri dishes containing a DMEM (Gibco, USA) nutrient medium enriched by 5% of fetal bovine serum (Sigma Aldrich, USA) and supplemented with antibiotics (streptomycin, 100 mg/mL; penicillin, 100 mU/mL, Aldrich, USA). Cells were propagated at 37 °C with 5% CO₂ in a humidified atmosphere (95% humidity). The culture medium was replaced every three days. Three replicate experiments have been carried out under identical conditions. Written informed consents were obtained from all volunteers before skin sampling, and the study was approved by the Ethics Committee of the Institute of Gerontology (approval number: 04/2018).

Cell proliferation assay

The effect of FX on the cell survival was determined 48 h after the addition of FX by the MTT test [19]. This assay quantifies viable cells by measuring the conversion of the yellow 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to purple formazan by mitochondrial succinate dehydrogenase, according to the manufacturer's instructions (Sigma Aldrich, USA). Cells were seeded in 96-well plates (5×10³ per cm²) at 15 × 10⁴ cells per plate and allowed to attach for 24 h. The medium was replaced with fresh medium containing DMSO and FX. The cells were treated with FX for 48 h. After that, the

medium was replaced again to avoid coloring effect of FX and 20 μL of MTT solution (5 mg/mL-1 PBS) was added to each well and incubated for a further 4 h at 37°C in 5% CO₂ and humidified air. Subsequently, 200 μL of pure 100% DMSO was added to solubilize the formazan crystals formed in cells. Absorbance values were read at 560/650 nm by Varioskan LUX Multimode Microplate Reader (Thermo Fisher Scientific, USA). Based on the absorbance of the cell samples, cell viability was measured. Cell viability was expressed as the amount of dye reduction in FX-treated cells relative to that of intact or untreated (DMSO) control cells. Each experiment was performed in triplicate and independently repeated at least four times.

Comet assay

The effect of FX on the level of DNA damage in hydrogen peroxide-exposed cells (180 μL of H₂O₂, 20 min) was determined using the Comet assay technique. For this assay, cells were seeded at concentrations 6000 per cm² in to the plastic Petri dishes (Cell Star BioTechnologies, Co Limited, Shanghai). After 24 h for adaptation, standard culture media were replaced by FX test combination. The concentration of FX in the DMSO/media solution was 5mcg/mL. This concentration was chosen since stimulating (hormetic) effect was obtained with this dose in MTT test. 0.2 % DMSO solution was used as a control. The Comet assay was performed by Klaude et al. [20] method with minor modifications. Briefly, after neutral lysis (2.5 M NaCl, 100 mM EDTA, 10 mM Tris and 1% of Triton X-100, pH 10) at 4 °C for 1 h, the slides were rinsed in TBE buffer (7.007 g Tris, 3.575 g Boric acid, 0,489 g EDTA, pH 8), 3 times for 5 min. Electrophoresis was conducted for 20 minutes at 25 V and 300 mA. All technical steps were conducted using very dim indirect light. After electrophoresis, the slides were gently removed and SYBR Green I (Sigma-Aldrich, St. Louis, MO, USA) (75 μL of a 20 $\mu\text{g/mL}$ solution) (30–40 μL) was added to each slide. DNA migration was analyzed on a microscope with fluorescence equipment (filter G-2A), and measured with a scaled ocular (Olimpus, blue LED, ext.460, ok.10). For the evaluation of DNA migration (total image length), 100 cells were scored from each slide. For pre-treatment with FX, cells were cultivated with FX during 72 h. Then cells were washed and exposed to H₂O₂ during 20 min. The FX-treated cells were compared with both intact control

cells and with H₂O₂-exposed negative control cells.

Statistical analysis

Shapiro–Wilk test was used to check the normality of variable distributions. MTT test data were normally distributed, so these data were analyzed by parametric ANOVA test with post-hoc multiple comparisons using Duncan test. Data for all Comet assay indices were non-normally distributed, so they were analyzed by non-parametric Kruskal–Wallis test and Dunn's median test for post-hoc multiple comparisons. All analyses were performed by Statistica 7.0 (StatSoft Inc., Tulsa, USA) software.

RESULTS

MTT test

MTT assay is commonly used for evaluating cell viability and cytotoxicity of potentially hazardous substances. We used this assay to assess potential cytotoxicity of FX. The results of MTT test are presented as the percentage of viable fibroblasts in the presence of FX at different concentrations compared to the DMSO control (Fig. 1). From the figure it is evident that high concentrations of FX (25, 50 and 100 mcg/mL) were toxic to fibroblasts in a dose-dependent manner. Treatment with low FX concentrations, unexpectedly, resulted in opposite (beneficial) effect on cell proliferation. Indeed, the proliferation rate of cells treated with low FX concentrations (3.13 and 6.25 mcg/mL) was significantly (about 20%) higher than that of the DMSO-control cells untreated with FX.

Comet assay

Comet assay is commonly used to determine the level of initial DNA damage (strand breaks, crosslinks or base damage) in individual cells exposed to DNA damage-inducing agents such as hydrogen peroxide. This assay allows to measure and compare the overall fluorescent intensity of the DNA in the nucleus with DNA that has migrated out of the nucleus. In our study, DNA damage level was assessed by Comet assay to determine double-strand breaks in control and H₂O₂-exposed cells with a total cell number of 100 per group. All Comet assay indices were strongly influenced by exposures applied [tail moment: Kruskal–Wallis test: H (2, N=300) = 96.8; tail DNA: H (2, N=300) = 47.8; olive tail moment: H (2, N=300) = 91.5; p < 0.001 for all]. Comet assay micrographs and Comet assay indices are presented in Fig. 2. As it is evident from the

figure, exposure to H₂O₂ resulted in significantly increased level of DNA damage in fibroblast cells. This unfavorable effect was completely diminished by using FX as a protective agent.

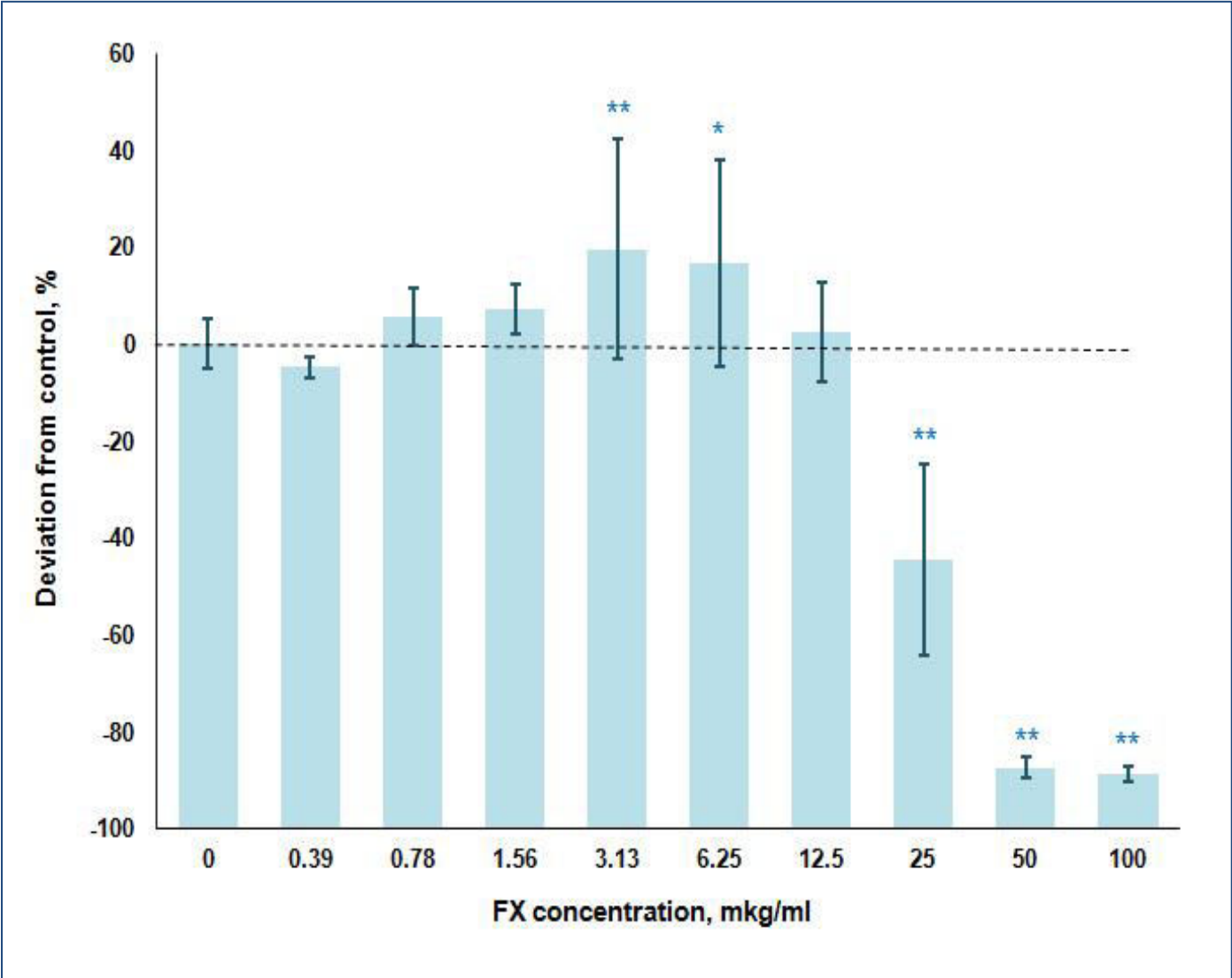
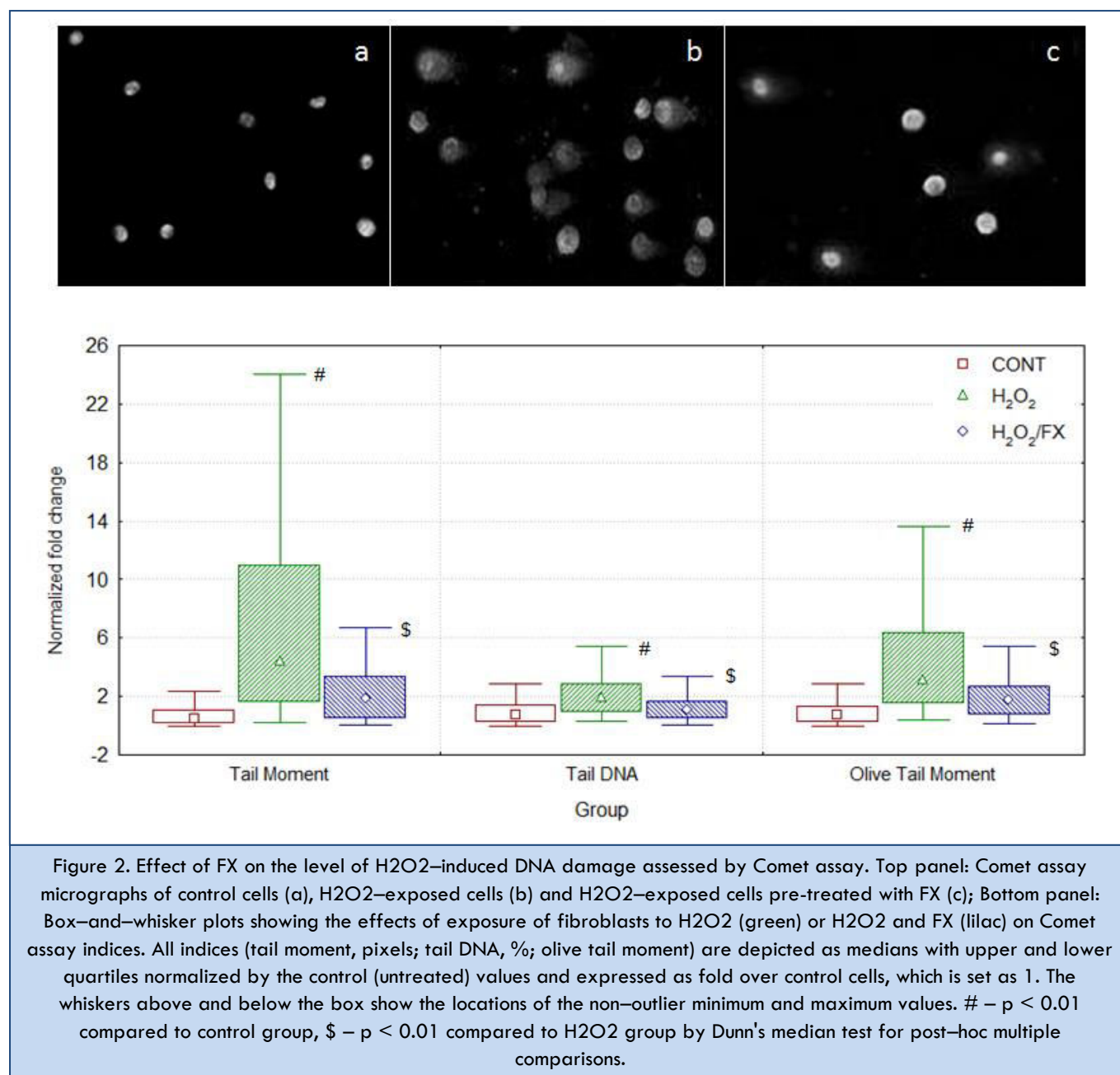


Figure 1. Percent deviation from control in cell viability (MTT test) in the DMSO–control fibroblasts and in fibroblasts treated with different concentrations of FX. The control level is indicated by a horizontal dashed line in the figure. Statistically significant differences from control are marked with asterisks (* – $p < 0.05$, ** – $p < 0.01$ by ANOVA post–hoc Duncan test).



DISCUSSION

With aging, oxidative stress and inflammation weaken repair mechanisms and cause collagen and elastic fiber breakdown in skin cells, thereby resulting in loss of volume and elasticity, wrinkling and pigmentation [21]. Some plant extracts were shown to be effective in protection against these processes [22]. In our study, FX exhibited cytoprotective effect against hydrogen peroxide-induced cell damage. Indeed, treatment of hydrogen peroxide-exposed fibroblast cells with FX resulted in inhibited Comet tail formation, suggesting that it may prevent the H₂O₂-induced DNA damage *in vitro*. These results

are similar to those of other studies where pretreatment with FX protected cultured human keratinocytes [23] and human fibroblasts [24] against UV-B radiation-induced oxidative damage, and also monkey kidney fibroblasts against H₂O₂-induced oxidative stress [25]. More recently, FX was also shown to exert cytoprotective effects against H₂O₂-induced oxidative damage in human hepatic L02 cells, which could be via the PI3K-dependent activation of Nrf2 signaling [26]. The capacity of FX to protect skin fibroblasts from oxidative stress

injury may likely be useful in developing treatment options aimed at combating skin photoaging.

Unexpectedly, treatment with FX caused beneficial effect on intact cells, which was evident from MTT assay. Indeed, a bell-shaped dose–response relationship was obtained in intact (unexposed to H₂O₂) cells. Such kind of relationship is peculiar to hormetic responses characterized by a high dose inhibition and a low dose stimulation. Hormetic response, in particular, is assumed to be a potential mechanistic explanation for revitalizing and health–promoting effects of herbal medicines [27]. The basic mechanisms involved in this kind of adaptive response include enhancement of DNA repair, free–radical scavenging, synthesis of heat shock proteins, apoptosis and compensatory cell proliferation, activation of cell–membrane receptors and secretion of various growth factors and cytokines [28, 29]; these processes are generally accompanied by adaptive changes in epigenetic regulation of gene expression [30]. Hormetic response is regarded as a plausible mechanism of anti–aging (life–extending) effects of FX in *Drosophila* [31]. Hormesis–induced substances ('hormetins') are believed to be potentially useful in combating skin aging [32]. For example, curcumin, a main component of the widely used spice turmeric, was found to be able to modulate wound healing ability of human skin fibroblasts in a biphasic dose response manner, being stimulatory at low doses and inhibitory at higher doses [33]. One other example of the hormesis-based skin care cosmetic is ginsenosides (active ingredients extracted from the roots of the Chinese herb Sanchi). This extract exhibited protective effects against facial wrinkles and also against other symptoms of facial skin aging, which could be due to hormetic mode of action of this compound by stress-induced synthesis of chaperones contributed to the repair and removal of damaged proteins [32]. Therefore, it is not surprising that hormetins (in particular, phytohormetins such as FX) are increasingly used in dermatology and cosmetology. One example is FX–based cosmetological composition such as Meso–Xanthin F199™, designed to revitalise mature skin. In an *in vitro* study, treatment of human skin fibroblasts with Meso–Xanthin F199™ resulted in an enhanced expression of genes encoding essential skin proteins (integrins), as well as GADD45A protein involved in DNA damage repair and regulation of transcription [34]. The therapeutic potential of FX for dermatological and

cosmetological applications has, however, not yet been sufficiently investigated and requires further investigation. In conclusion, data obtained in present study are suggestive of a therapeutic potential of FX for dermatological and cosmetological applications.

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Compliance with Ethical Standards

Ethical Statements

The study protocol was approved by Ethical Committee of the Institute of Gerontology (approval number: 04/2018)

Conflict of Interest

The authors declare that they have no conflict of interest.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

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