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FLAX OIL FROM TRANSGENIC *LINUM USITATISSIMUM* SELECTIVELY INHIBITS *IN VITRO* PROLIFERATION OF HUMAN CANCER CELL LINES

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Abstract: Emulsions made of oils from transgenic flaxseeds significantly decreased *in vitro* proliferation of six tested human cancer cell lines in 48-h cultures, as assessed with the standard sulforhodamine assay. However, the emulsions also increased proliferation rate of normal human dermal fibroblasts and, to a lower extend, of keratinocytes. Both inhibition of *in vitro* proliferation of human cancer cell lines and stimulation of proliferation of normal dermal fibroblasts and keratinocytes were especially strong with the emulsion type B and with emulsion type M. Oils from seeds of transgenic flax type B and M should be considered as valuable adjunct to standard cytostatic therapy of human cancers and also could be applied to improve the treatment of skin lesions in wound healing.

Keywords: transgenic flax seeds, oil emulsions, *in vitro* proliferation, cancer cells, normal skin cells

Flax (*Linum usitatissimum* L.) - an annual plant, primarily cultivated for industrial purposes, as a source of fibers and oil, was recently genetically modified in order to enhance wound healing properties of the fibers (1-3).

As a result, new types of genetically modified flax provide stable overexpression of enzymes for polyhydroxybutyrate (PHB) synthesis (1) and enzymes of phenylpropanoid pathway (2). New flax fibers overexpressing those genes accumulate components like phenolic acids and flavonoids with antioxidant activity (2). Flax oil is recommended as a component of balanced human diet in preventing diverse human illnesses such as heart disease (4) metabolic syndrome, insulin resistance and diabetes mellitus (5, 6), breast cancer (7, 8) and regular ingestion of flax oil attenuates oppresive menopausal symptoms (9) and alleviates digestive tract irregularity, diarrhea and constipations (10, 11). Flaxseeds oil supplementation decreases oxidative stress, and probably *via* this mechanism reduces inflammation and insulin resistance (5). Flaxseeds oil is also used in dermatology and cosmetology as an ingredient of ointments, emulsions and lotions applied topically on skin. Previously, in pre-clinical studies, it was documented that transgenic flax plants which contained increased level of polyphenolic compounds significantly enhanced healing of skin wounds (12). In a number of *in vitro* studies flax fabrics and emulsions made of oils from genetically modified flax seeds significantly enhanced proliferation of human primary dermal fibroblasts (13-15) and keratinocytes (14).

A wide use of flaxseed oil in human diet and frequent topical administration in dermatology impose careful evaluation of flax oils influence on *in vitro* proliferation of human cancer cells. The impact of emulsions from flaxseed oils on proliferation rate of six human cancer cell lines both from genetically modified plants (M, B and MB) and from natural Nike cultivar is shown in the present paper. Additionally, the influence of tested emulsions on proliferation of human normal skin fibroblasts and keratinocytes cultures is also included. Activities of

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the tested emulsions of oils from transgenic flaxseeds and from nontransgenic flaxseeds (Nike) were compared by means of the multicriterial analysis.

EXPERIMENTAL

Chemicals

Sulforhodamine (SRB), Trizma-base, trichloroacetic acid (TCA), Tween 80 and glycerol were purchased from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS), 2 mM L-glutamine solution, antibiotics solution: penicillin (100 U/mL) and streptomycin (0.1 mg/mL), trypsin EDTA solution were obtained from Lonza (Verviers, Belgium). Cell culture plastic flasks (75 cm^2) as well as 24-wells culture plastic plates were purchased from Lonza (Verviers, Belgium). Phosphate buffered saline (PBS), 0.4% trypan blue solution and all other chemicals were obtained from POCH (Gliwice, Poland). Soybean lecithin (Lipoid S75) was from Lipoid (Ludwigshafen, Germany).

Cells

Normal human dermal fibroblasts (NHDF), Normal Human Epidermal Keratinocytes (NHEK), both from adult donors, were purchased from Lonza (Verviers, Belgium).

Human cancer cell lines: A549 (pulmonary basal cell alveolar adenocarcinoma), A431 (epidermoid carcinoma), MCF7 (breast cancer), CCRF/CEM (T cell lymphoblastoid acute leukemia), LOVO (colon adenocarcinoma), were obtained from ATCC (American Type Culture Collection, Manassas, VA, USA). The doxorubicin resistant cell subline LOVO/DX was derived from the original drug-sensitive LOVO cell line by 3-week cultivation of cells in the presence of low concentration of doxorubicin.

Plants

The transgenic flax have been generated by transforming *Linum usitatissimum* L., fibrous cultivar Nike as described previously (1, 3). The plant exhibits stable overexpression of enzymes for polyhydroxybutyrate (PHB) synthesis and enzymes of phenylpropanoid pathway, therefore transgenic flax fibers and flaxseeds accumulate components like phenolic acids and flavonoids with antioxidant activity (16-18) B, M and MB transgenic plants as well as fibrous Nike cultivar were grown in fields near Wrocław in 2015 and were harvested after 4 months.

Methods

Preparation of oil emulsions

Preparation of oil from flaxseeds and emulsions made of oils followed the procedurę described previously (18, 19). Briefly, soybean lecithin and Tween 80 were mixed with flax oil and then an aqueous phase containing glycerol was added and mixed vigorously. The sample was then sonicated using Microson ultrasonic cell disruptor (Misonix Inc., Farmingdale, NY, USA) for 10 min at 4 W. The sonicated preparations were filtered through 0.22 μm filters. All the emulsion samples were prepared at room temperature. The final concentrations of the chemicals in emulsion were 1% lecithin, 2.5% flax oil, 2.5% Tween 80 and 2.5% glycerol (20). Content of total phenolics, sterols, lipid soluble antioxidants and fatty acid in the tested emulsions were established according to the methods described previously (20).

Cell culture conditions

Cells were grown in the culture media recommended by cell line supplier. Before the test, adherent cells were detached with the trypsin EDTA solution, washed with PBS, spun down, counted, stained with a 0.4% solution of trypan blue, and inspected under a microscope for cell viability. Then, cells were plated on 24-well plastic culture plates (2×10^3 cells/well) and incubated at 37°C in a CO_2-incubator for 24 h, afterwards, the tested emulsions were added, assuming 2.5% (2.5 g/100 mL) content of oil in the final emulsion (20), and the cultures were incubated for 48 h in CO_2-incubator at 37°C. Then, cells were harvested and intended for cell proliferation test.

Determination of cell density/cell proliferation

Cell density/cell proliferation was estimated with the sulforhodamine B (SRB)-colorimetric assay (21). Briefly, cell cultures were fixed with cold TCA (final concentration 10% (w/v) in cultures of adherent cells for 1 h at 4°C, then washed four times with tap water and air-dried at room temperature (20-25°C). A mildly acidic SRB solution (0.4% dye solution in 1% acetic acid) was added to each well for 30 min at 25°C and then, unbound stain was removed by rinsing with an aqueous solution of 1% (v/v) acetic acid. Culture plates were then allowed to dry at room temperature. The SRB bound to the intracellular proteins was dissolved in 10 mM Trizma-base solution (pH 10.5) for 10 min on a gyratory shaker and absorbance of the SRB solution was estimated at 540 nm in a Victor 2 microplate reader (Perkin-Elmer, MA, USA).

Statistical analysis

The results estimated in cultures carried out with the presence of the tested emulsions were compared to the relative control cultures (cells cultured without the emulsions) with the standard paired *t* test .

The multicriterial analysis (MCA) was calculated according to literature data (22) with own modifications (20). Briefly, the results obtained with six *in vitro* tests for emulsion concentration of 1.25 mg/mL were compared to the relative control cultures [E/E_0] and the statistical distances between the expected (exp.) and observed (obs.) results were calculated with the standard formula: $b = (exp. - obs.)^2 / exp.$ The expected values in each test (decreased proliferation of cancer cells and increased proliferation of fibroblasts and keratinocytes) were assumed the most favorable results (indices of importance) of the tested emulsions. Results of the calculation, expressed as 1/b ratios, were then multiplicated by the indices of importance and yield the final outcome of the calculation. Finally, the results of the MCA procedure of compared emulsions were obtained according to equation: $M = \Sigma\ 1/b$.

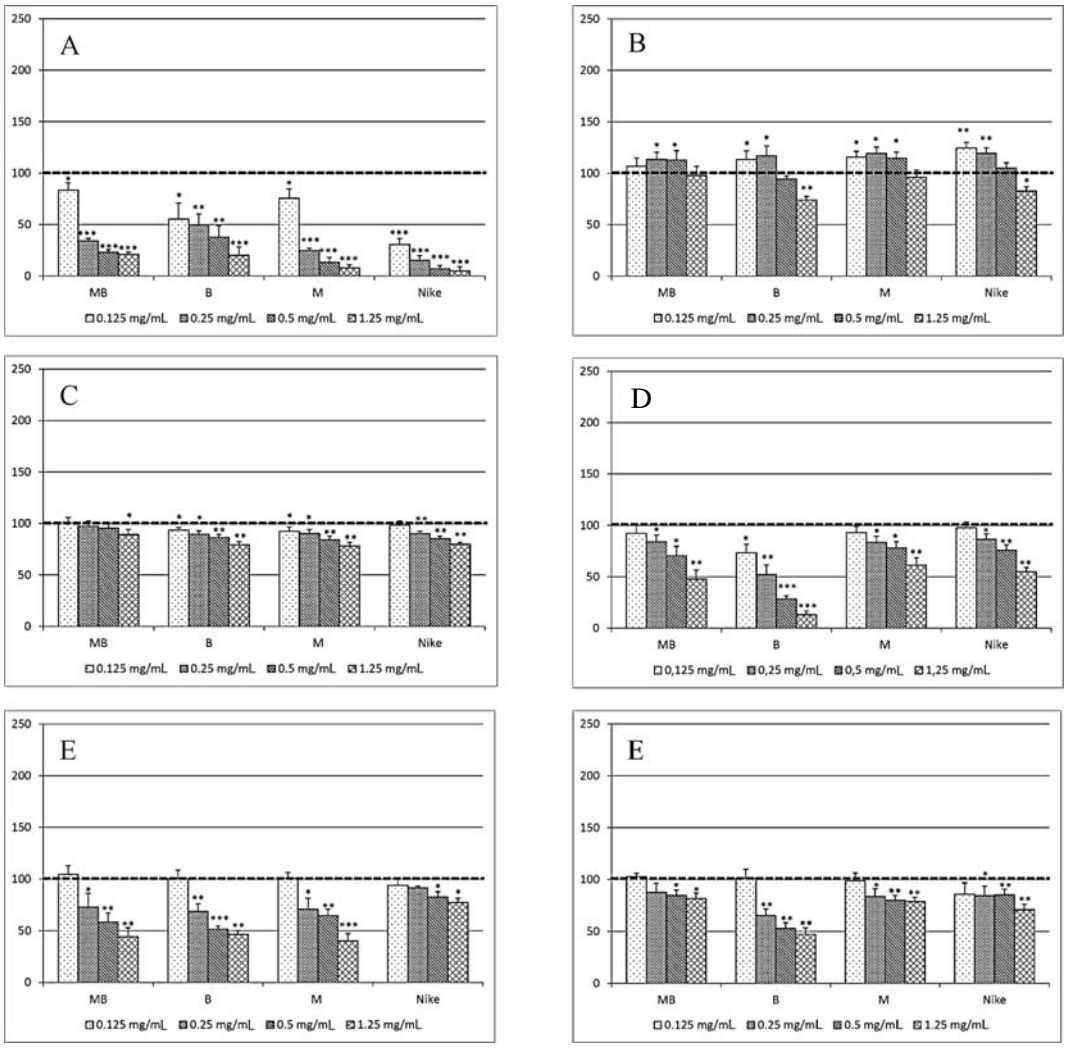

Figure 1. Impact of the tested emulsions made of oils from flaxseeds on *in vitro* proliferation of human cancer cell lines: A - CCRF/CEM (T cell lymphoblastic leukemia), B - A549 (lung alveolar adenocarcinoma), C - A431 (epidermoid carcinoma), D - MCF7 (breast cancer), E - LOVO (colon adenocarcinoma cells with average sensitivity to doxorubicin) and F - LOVO/DX (colon adenocarcinoma cells resistant to doxorubicin), compared to proliferation of the relative controls (cells cultured without tested emulsions; C = 100); (mean ± SD, n = 5). Statistical significance was calculated with the paired *t*-test (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$

RESULTS

Impact of the tested emulsions on *in vitro* proliferation of six human cell tumor lines is shown in histograms in Figure 1.

The emulsions from flaxseeds oil significantly decreased *in vitro* proliferation of the tested human tumor cell lines, the decrease was especially strong at the highest tested dose of emulsions (corresponding to flax oil content of 1.25 mg/mL). At this concentration, the strongest effect on MCF7 (breast cancer) cell proliferation was caused by emulsion type B (decrease by 85%), on CCRF/CEM (T cell leukaemia) – emulsions B and M (by 75 and 85%), LOVO (colon cancer) – the emulsions B and M (by 53 and 60%, respectively), A431 (epidermoid carcinoma) – emulsions B and M (by 20%) and A549 (bronchoalveolar adenocarcinoma) – the emulsion type B (by 28%). The effect of flaxseeds oil emulsions on A549 cell proliferation was different in various concentrations of the emulsion: at lower concetration (0.125-0.5 mg/mL) increase of A549 cell proliferation by 13-20% was estimated both with the emulsions from transgenic flaxseeds oils and with non-transgenic flaxseeds oil (Nike). At the highest tested concentration (1.25 mg/mL) emulsions M and MB did not influence significantly on A549 cell proliferation, whereas emulsions B and Nike markedly decreased (by 20 and 27%, respectively) proliferation of cancer cells. The effect of flaxseeds oil emulsions on A549 cell proliferation needs further studies. Importantly, the decrease of cancer cell proliferation was strong also in the case of drug-resistant cell subline LOVO/DX wherein the decreasing effect of emulsions B and M on cell proliferation was only slightly weaker than in parental, drug-sensitive cell line (LOVO); at the concentration of of 1.25 mg/mL proliferation of LOVO (drug-sensitive) decreased by 53 and 60%, respectively, of LOVO/DX (drug-resistant) by 52 and 20%, respectively. Also, it should be stressed that this inhibitory effect was markedly stronger in the cases of the emulsion made of oil from transgenic plants flaxseeds than in the case of nontransgenic counterpart (Nike).

In paralel series of experiments the effect of tested emulsions on proliferation of normal human dermal fibroblasts (NHDF) and keratinocytes (NHEK) was evaluated with the same SRB method. The results are shown in histograms in Figure 2.

As shown in Figure 2, the tested emulsions marekedly enhanced proliferation of normal human dermal fibroblasts - at the concentrations of 0.25-0.5 mg/mL, even by more than 50% (emulsions M and MB) and more than twice as high with B emulsion as the control culture (without the tested emulsions). At higher concentration of the emulsions (1.25 mg/mL), the stimulatory effect was significantly weaker and even small decrease of fibroblast proliferation was observed with M and Nike emulsions (decrease of proliferation by 10%). However, in the case of B emulsion, fibroblasts proliferation was still elevated (at 1.25 mg/mL) by about 50%, when compared to the control cultures.

Normal human keratinocytes proliferation was significantly elevated (by10-30%) in cultures with MB emulsion. The stimulatory effect was markedly weaker with M and Nike emulsions (increase by 2-5%) and with B emulsion (increase by 1-2%).

The tested emulsions were compared with the multicriterial analysis in the aspect of their potency to decrease cancer cell proliferation and to increase proliferation of normal skin fibroblasts and keratinocytes. The assumed ranking and rating criteria should fullfil

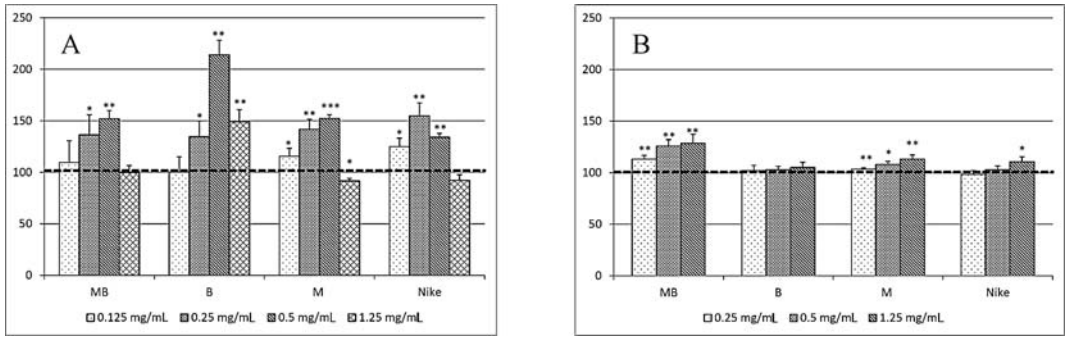

Figure 2. Impact of the emulsions made of oils from flaxseeds on proliferation of normal human dermal fibroblasts (A) and keratinocytes (B) compared to relative controls (cultures without tested emulsions; C = 100); (mean ± SD; n = 5). Statistical significance was calculated with the paired *t*-test (*p < 0,05; **p < 0,01; ***p < 0.001)

Table 1. Impact of the tested emulsions made of oils from flaxseeds on proliferation of human cancer cell lines (A-F) and normal skin cells (G, H) compared by means of the multicriterial analysis (MCA).

Cell culture		Expected effect	Indices of importance	Tested emulsions			
				Nike	M	B	MB
A	CCRF/CEM	↓	0.05	0.004	0.002	0.000	0.000
B	A549	↓	0.05	0.010	0.004	0.037	0.003
C	A431	↓	0.15	0.126	0.188	0.141	0.032
D	MCF7	↓	0.15	0.001	0.001	0.011	0.001
E	LOVO	↓	0.10	0.002	0.028	0.012	0.016
F	LOVO/DX	↓	0.20	0.007	0.005	0.047	0.004
G	keratinocytes	↑	0.15	0.015	0.018	0.011	0.063
H	fibroblasts	↑	0.15	0.003	0.003	0.041	0.004
Total (sum of the results in columns)			1.0	0.168	0.248	0.300	0.124

Table 2. Content of estimated compounds in the emulsion from control non-transgenic (Nike) and transgenic plants (B, M and MB); mean ± SD, n = 5.

Estimated compound	Flaxseeds type as a source of oil emulsions			
	NIKE	M	B	MB
Lutein (mg/100 mL)	0.03 ± 0.003	0.03 ± 0.003	0.04 ± 0.006	0.04 ± 0.01
γ-Tocopherol (mg/100 mL)	0.97 ± 0.16	0.92 ± 0.09	0.87 ± 0.02	0.82 ± 0.14
Total phenolics (μg/100 mL)	6.29 ± 0.65	6.79 ± 0.049	7.17 ± 0.58	7.53 ± 1.20
β-Sitosterol (μg/100 mL)	138.90 ± 4.07	131.14 ± 2.35	147.96 ± 1.33	133.89 ± 3.00

the following expectations 1. decrease of drug-resistant cancer cell proliferation (LOVO/DX); 2. inhibition of epidermal cancers (A431, MCF7) proliferation; 3. increase of fibroblast and keratinocyte proliferation. Relatively higher indices of importance were confered for these rating criteria. The multicriterial analysis was carried out with the results obtained at emulsions' concentration of 1.25 mg/mL. Results of the calculation are given in Table 1.

The analysis proved that the emulsion type B exhibited outstanding effect on proliferation of six tested cancer cell lines (decrease) and on skin cells (increase), the calculated total favorable effect of the B emulsion was almost 2 times as high as that of non-transgenic emulsion Nike. Also the emulsion type M revealed total favorable effect higher by 50% when compared to emulsion from non-transgenic Nike flaxseeds. Distinctly, the effect of the emulsion type MB as calculated with the multicriterial analysis provided total effect by 27% lower than the total effect of Nike emulsion.

Contents of total phenolics, sterols and lipid soluble antioxidants in the tested emulsions are given in Table 2.

As could be seen in Table 2 the tested oil emulsions from flaxseeds of transgenic plants compared to the emulsion from non-transgenic flax seed (Nike) contained markedly higher accumulation of β-sitosterol (emulsion type B), total phenolics (emulsions: MB, B, and M) and lutein (emulsion type B and MB), but content of γ-tocopherol in transgenic and non-transgenic (Nike) flaxseeds did not differ. Also, accumulation of both saturated and non-saturated fatty acids, both n-3 and n-6 did not differ significantly between the tested emulsions (data not shown).

DISCUSSION

Results obtained in this paper show that the presence of tested emulsions in cultures of human tumor cell lines lead to significant decrease of can-

cer cell proliferation. However, marked increase of proliferation of normal human dermal fibroblasts and of skin keratinocytes was estimated in the parallel series of experiments with the emulsions. Importantly, the emulsions were almost equally as active against the drug-resistant cell (LOVO/DX) as in the drug-sensitive parental cell line (LOVO). The tested emulsions were compared with the multicriterial analysis with their potency to decrease cancer cell proliferation and to increase proliferation of normal skin fibroblasts and keratinocytes. The assumed ranking and rating criteria comply with the following expectations 1. decrease of drug-resistant cancer cell proliferation (LOVO/DX); 2. inhibition of epidermal cancers proliferation (A431, MCF7); 3. increase of fibroblast and keratinocyte proliferation. Relatively higher indices of importance were conferred on these rating criteria. The analysis proved that the emulsion type B exhibited outstanding effect on proliferation of six tested cancer cell lines (decrease) and on skin cells (increase). The calculated total favorable effect of the B emulsion was almost two times as high as that of non-transgenic emulsion Nike. Also emulsion type M revealed favorable total effect in the analysis, being higher by 50% when compared to emulsion from non-transgenic Nike flaxseeds. Distinctly, the effect of emulsion type MB provided total effect by 27% lower than that of Nike emulsion.

Analysis of chemical composition showed that the emulsions from transgenic flaxseeds oil (B, M and MB) included relatively higher content of total phenolics, sterols (B) and lutein (B, MB) when compared to the emulsion from non-transgenic flaxseeds (Nike). Outstanding effects of emulsion B on both cancer cell cultures (inhibition of proliferation) and on skin cell cultures (increased proliferation) could be explained, at least in part, by increased content of β-sitosterols and total phenolics in this oil emulsion.

One could hypothesize that the effect of the tested emulsions on *in vitro* proliferation of cancer cells and normal skin cells could be dependent on their interference with cellular metabolic pathways, especially with lipid metabolic pathways.

Growing body of literature data emphasize the fact that cancer cells are strongly dependent on lipid metabolism and exhibit significant alterations in lipid metabolic enzymes' activity - lipid metabolism reprogramming (23). Lipid reprogramming is intended to support cancer cells' requirements for high cholesterol content in cells (23) that need to be finely regulated (24). Cellular control of lipid metabolism includes the liposensor function of the nuclear receptor LXR β (liver X receptor β). Activated LXR induces transcription of genes involved in metabolism and cellular transport of cholesterol (24) and also decreases cell proliferation (stimulates $p27^{kip1}$ protein, one of the inhibitors of cell-cycle progression from G1 to S phase) (25). Published reports of anti-proliferative effects of LXR ligands on various human cancers suggest that LXR could be potential target in cancer prevention and treatment (26). LXR is activated by oxysterols (oxidized derivatives of cholesterol) and also by various synthetic agonists, dietary phytosterols, particularly β-sitosterol (27), and several flavonoids (28). The emulsions made of oils from transgenic flaxseeds included elevated content of both β-sitosterol and total polyphenolics, therefore the activation of LXR in cancer cells is likely mechanism decreased cancer cell proliferation in the presence of the emulsions. The highest content of β-sitosterol and polyphenolics was estimated in the emulsion type B, and was correlated with their strong decrease of cancer cell proliferation.

Among human cancer cell lines evaluated in this paper, the pulmonary adenocarcinoma cells (A549) highly express LDLR - cell surface recetor for low density lipoprotein (29). As was established in the SRB assay, A549 cells increased their proliferation rate in the presence of low and moderate doses of the emulsions, whereas decreased proliferation was observed exclusively at the highest concentration of the emulsions (1.25 mg/mL). Perhaps A549 cells are able to uptake higher portion of lipids and sterols supplied with the tested emulsions (LDLR-mediated endocytosis), thereby cell energy metabolism and proliferation are enhanced in these range of the emulsion concentration. However, at higher concentration of lipids (higher doses of the tested emulsions added to cell cultures), by-products of enhanced lipolysis (oxysterols) accumulate and lead to prevailed decrease of A549 cells proliferation (*via* LXR-mediated pathways).

Likewise, the distinct impact of flaxseed oil emulsion on normal skin cells and on cancer cells could be explained by marked differences in their lipid pathways. Since the normal human dermal fibroblasts and keratinocytes express high level of LDLR (30), they uptake lipids supplied with flaxseed oil emulsions, metabolize them in normal, physiological lipolytic pathways, and achieved higher level of metabolic energy, which increases their vitality, proliferation rate of fibroblasts and differentiation of keratinocytes (30). However, the inhibitory effect of the emulsions on fibroblasts' proliferation was observed at the highest tested con-

centration of the emulsions, probably due to the LXR pathways activation. By contrast, cancer cells, which display changed/reprogrammed lipid pathways (23-25) are not able to correctly manage lipids of flaxseeds oil emulsions, and abundance of oxysterols and other by-products of aberrant lipolysis early leads to activation the LXR (thereby inhibits proliferation). Nevertheless, attention should be drawn to the influence of flaxseeds oil emulsions on content of cytostatic drugs in cancer cells, since it was documented in the literature that activated LXR induces transcription of several efflux proteins and would decrease cancer cells' sensitivity to standard anticancer drugs (24).

Flaxseeds oils from transgenic plants could be considered as valuable adjunct to standard cytostatic drugs in the treatment course of human cancers and also can be used to improve skin wound healing. Further *in vitro* studies should be focused on evaluation of intracellular content of the cytostatic drugs in cancer cells cultured in the presence of the tested emulsions.

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