with a low electrical conductivity of 0.14 S/m (10mM KH_2PO_4/K_2HPO_4, 1mM $MgCl_2$, and 250mM sucrose; pH 7.4). The cell suspension was pulsed in a cuvette with 2 aluminum plate electrodes (4 mm space between electrodes) with electrical field strength up to 3,000 V/cm using 8 pulses of 100 µs duration. Rectangular electrical pulses were delivered by an electroporator ECM 830 (BTX Harvard Apparatus; Syngen Biotech, Wrocław, Poland). In the case of the cells being treated with tamanu oil and undergoing EP, electrical fields with the intensity of 800 V/cm and 1,000 V/cm were used. Following pulsation, the cells were left for 10 min at 37°C, centrifuged, resuspended in fresh cell culture medium, and reseeded for a viability assay and immunocytochemistry (ABC method).

Cellular viability

Cellular viability was studied by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (In Vitro Toxicology Assay; Sigma-Aldrich), which assesses the mitochondrial redox activity as an indicator of the cellular proliferation potential. The MTT assay was performed 24 h and 72 h post-treatment, according to the manufacturer's protocol. The absorbance was measured at 570 nm using a multiwell plate reader (EnSpire Multimode Reader; Perkin Elmer Polska, Kraków, Poland). The assay was performed independenly 3 times using 3 repetition samples, and the mean values and standard deviation (SD) of combined results were calculated.

Immunocytochemical ABC staining of collagen III and mitochondrial superoxide dismutase

The expression of selected proteins was examined by the immunocytochemical Avidin-Biotin Complex (ABC) method. After fixation in 4% paraformaldehyde, the samples were permeabilized and blocked by incubation with 0.1% Triton X-100 (Sigma-Aldrich) in phosphate-buffered saline (PBS). The protein expression was visualized with a polyclonal antibody COL-III (1:100, anti-collagen III) and anti-SOD2 (Santa Cruz Biotechnology, Dallas, USA). Immunocytochemical staining was performed with ImmPRESS Universal Reagent (Vector Laboratories, Burlingame, USA).

Results

Cellular viability

The influence of tamanu oil, electric field intensity and the combination of both on cellular viability was assessed using MTT assay. The investigation indicated that using the tamanu oil increased viability in human fibroblast cell line, stimulating the proliferation of NHDF cells at every dilution, in contrast to HVF cells, in which higher viability

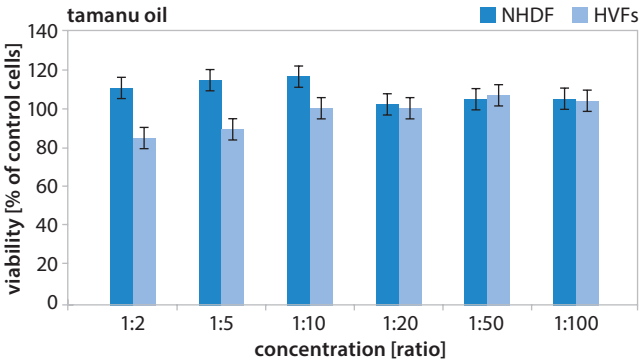

Fig. 1. The effect of *Calophyllum inophyllum* extract on the viability of normal human dermal fibroblast (NHDF) cells and cells from primary cell cultures (human vaginal fibroblasts – HVFs)

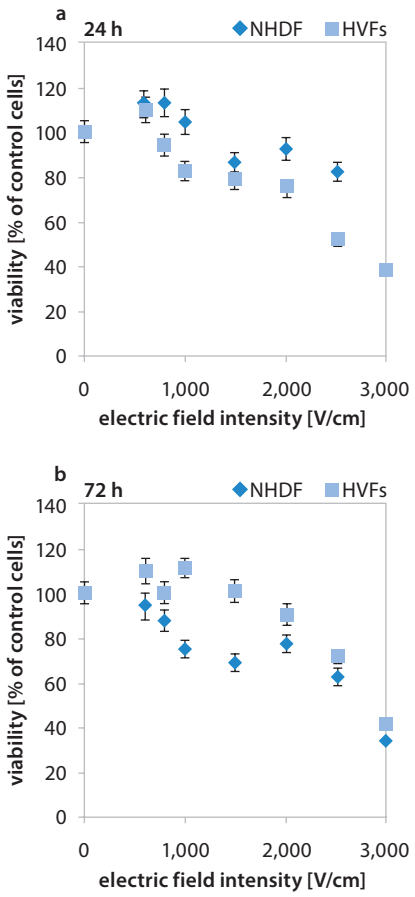

Fig. 2. The impact of pulsed electric field on human fibroblast (normal human dermal fibroblasts (NHDFs) and human vaginal fibroblasts (HVFs)) viability a) 24 h and b) 72 h after exposition

was observed only for further dilutions (1:20; 1:50; 1:100) (Fig. 1). From the experiment in which different currents were used, we selected the electric field of 800 V/cm intensity as the most beneficial for further experiments with the application of the oil extract from *C. inophyllum* on both cell lines (Fig. 2). Surprisingly, combining EP with tamanu oil resulted in a much greater increase in cell proliferation of HVF cells compared to NHDF cells. The highest proliferation was observed at 800 V/cm electrical field intensity and 1:10 dilution of tamanu oil (Fig. 3).

Immunocytochemical ABC reaction: collagen III and MnSOD

The results of immunostaining with anti-collagen III and anti-mitochondrial superoxide dismutase 2 (anti-MnSOD2) are presented in Tables 1–4 and in Fig. 4 and 5. An increase in collagen III expression was noted only 24 h post-incubation with tamanu oil (Table 1, Fig. 4). The most intense immune reaction was observed after incubation with tamanu oil at a dilution of 1:10 with application of an electric field of 800 V/cm intensity (c.a. 70% for NHDF and 30% for HVF) after 24 h of incubation. After 72-hour incubation with tamanu oil in combination with EP, the level of COL-III insignificantly decreased in NHDF cells and increased in primary HVF cells (Table 1, Fig. 4). Contrary to collagen III, the MnSOD expression indicated more intense staining reaction. The intensity of anti-MnSOD reaction increased proportionally with the increasing electric field intensity (Tables 1,2, Fig. 5) in NHDF cells. The highest expression was noted after a 24-hour incubation for cells treated only with tamanu oil and for cells treated with oil-EP combination (90% and 100%, respectively) (Table 1, Fig. 5). The expression of this antioxidant enzyme was lower after 72 h than 24 h post-treatment for cells treated only with tamanu oil and for the combined treatment (Table 2, Fig. 5). In HVF cells, the expression of MnSOD also increased at the higher EP parameters after 72 h in 100% of cells (Tables 3,4).

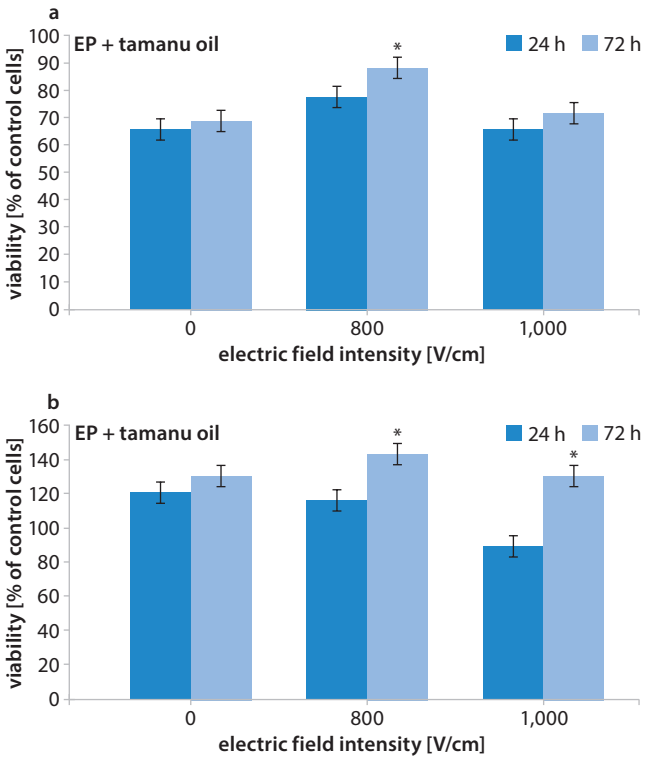

Fig. 3. The influence of *Calophyllum inophyllum* extract combined with electroporation (EP) on human fibroblasts viability 24 h and 72 h post-treatment in: a) normal human dermal fibroblasts (NHDFs) and b) human vaginal fibroblasts (HVFs)

Discussion

Prognosis of vaginitis in many cases is promising, but most of the infections are not completely cured and, therefore, reoccur. The recurring vaginal infection might lead to chronic infections and scarring,[10] but in most cases when suitably treated do not cause permanent problems. Conversely, untreated vaginal infections can spread to other pelvic structures and result in chronic diseases.[11] In such cases, another course of treatment is necessary. Natural compounds are commonly applied in many diseases of the human body, against both the precancerous state and cancer. However, it is commonly known that some of these compounds can be used in other non-cancer diseases. As an example can serve tropical tamanu oil, an extract from *C. inophyllum*. The properties of this oil have been known for a long time, especially for healing wounds. Women frequently use tamanu oil to achieve healthy, clear skin, as it helps to clear acne and scars. These compounds

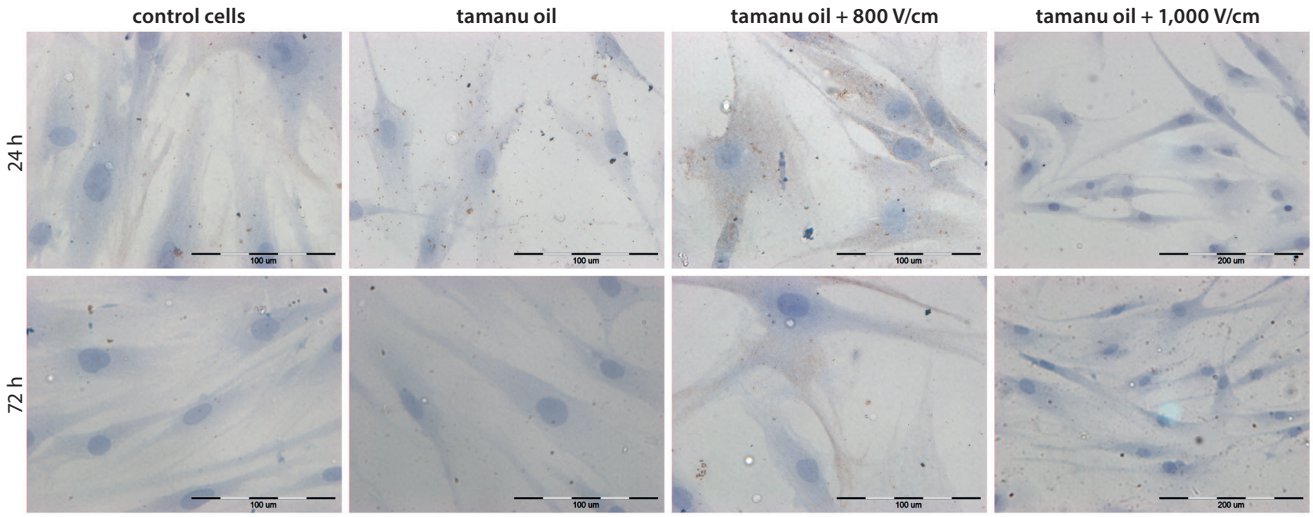

Fig. 4. The immunocytochemical staining reaction of collagen III in normal human dermal fibroblasts (NHDFs) 24 h and 72 h post-treatment

also have anti-inflammatory properties reducing swelling of rashes, insect bites and sunburns. Tamanu oil additionally possesses significant antimicrobial, antibacterial and antifungal qualities.[11] In this investigation, we examined the effect of tamanu oil on the proliferation of human established cell line fibroblast line (NHDF) and human primary fibroblast isolated from vagina (HVF). Additionally, we used EP in order to improve the transport of the examined compound. However, we observed an increase in proliferation in both human fibroblast cell lines

Table 1. The immunocytochemical evaluation of collagen III and mitochondrial superoxide dismutase (MnSOD) proteins in established normal human dermal fibroblast (NHDF) cells 24 h after incubation with tamanu oil and after electroporation (EP) combined with examined compound for 800 V/cm and 1,000 V/cm electric field intensity

Sample group	Tamanu oil concentration	EP (V/cm)	Intensity of immunoreaction	
			collagen III	MnSOD
Control group	no tamanu oil used	no EP applied	<5% +	55% +/++
Group I	1:10	0	50% +	90% ++
Group II	1:10	800	75% ++	100% ++
Group III	1:10	1,000	<5% +	100% ++

Table 2. The immunocytochemical evaluation of collagen III and mitochondrial superoxide dismutase (MnSOD) proteins in established normal human dermal fibroblast (NHDF) cells 72 h after incubation with tamanu oil and after electroporation (EP) combined with examined compound for 800 V/cm and 1,000 V/cm electric field intensity

Sample group	Tamanu oil concentration	EP (V/cm)	Intensity of immunoreaction	
			collagen III	MnSOD
Control group	no tamanu oil used	no EP applied	<5% +	70% +/++
Group I	1:10	0	<5% –/+	80% ++
Group II	1:10	800	35% +/++	85% ++
Group III	1:10	1,000	10% +	95% +++

Table 3. The immunocytochemical evaluation of collagen III and mitochondrial superoxide dismutase (MnSOD) proteins in primary human vaginal fibroblast (HVF) cells 24 h after incubation with tamanu oil and after electroporation (EP) combined with examined compound for 800 V/cm and 1,000 V/cm electric field intensity

Sample group	Tamanu oil concentration	EP (V/cm)	Intensity of immunoreaction	
			collagen III	MnSOD
Control group	no tamanu oil used	no EP applied	–	45% ++
Group I	1:10	0	10% ++	100% ++
Group II	1:10	800	30% ++	90% ++
Group III	1:10	1,000	35% ++	100% +++

Table 4. The immunocytochemical evaluation of collagen III and mitochondrial superoxide dismutase (MnSOD) proteins in primary human vaginal fibroblast (HVF) cells 72 h after incubation with tamanu oil and after electroporation (EP) combined with examined compound for 800 V/cm and 1,000 V/cm electric field intensity

Sample group	Tamanu oil concentration	EP (V/cm)	Intensity of immunoreaction	
			collagen III	MnSOD
Control group	no tamanu oil used	no EP applied	–	100% +++
Group I	1:10	0	15% ++	100% ++/+++
Group II	1:10	800	40% ++	100% +++
Group III	1:10	1,000	75% ++	100% +++

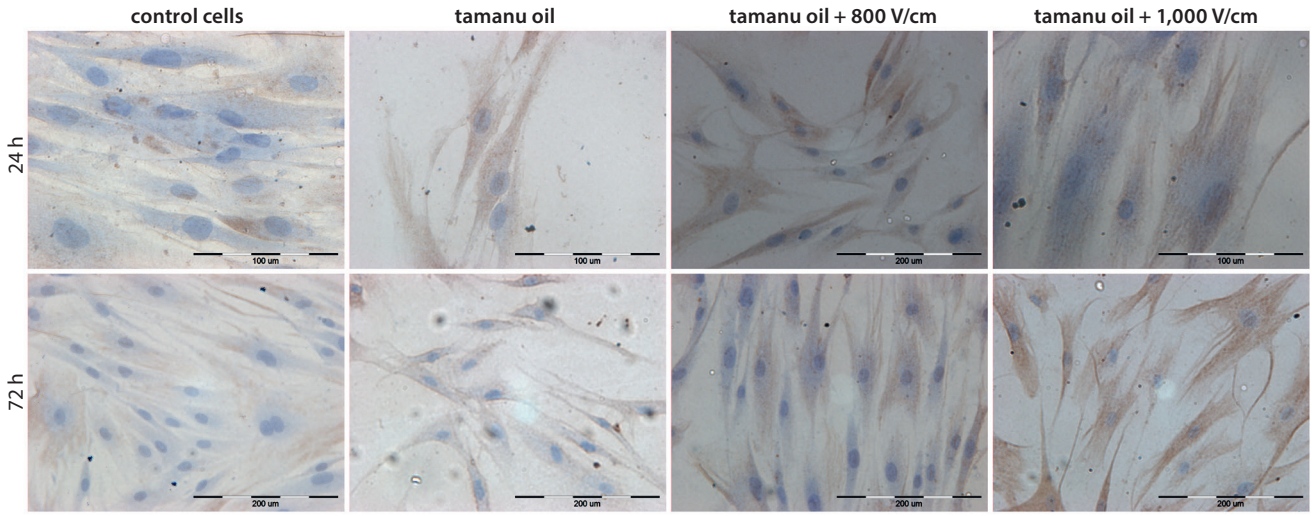

Fig. 5. The immunocytochemical staining reaction of superoxide dismutase (anti-MnSOD) in normal human dermal fibroblasts (NHDFs) 24 h and 72 h post-treatment

only for the electric field strength of 800 V/cm. A higher electric field intensity caused a similar effect in the control non-treated cells. Our results indicated that it stimulated cell proliferation, which suggests that tamanu oil could become a promising agent for human fibroblast re-growth. Therefore, it could help in developing a new, more effective method in vaginitis treatment. During the healing phase (so-called "proliferation") collagen and elastin fibers are formed to build up a strong and flexible skeleton, which is then filled by proteoglycan molecules and glycoproteins that combine matrix and healing cells in the wound.[12,13] In order to verify the healing properties of the chosen natural oil, the expression of selected extracellular matrix protein (collagen III) was examined. An increase of collagen III expression was noted 24 h and 72 h after incubation with the extract from *C. inophyllum* seeds. The highest expression was observed after incubation with tamanu oil at a dilution of 1:10, with a pulsed electric field (800 V/cm). Higher (1,000 V/cm) EP parameters induced decrease of immunoassayed reaction with anti-collagen III, suggesting that the higher EP parameters in combination with tamanu oil do not support collagen expression in NHDF and HVF cells. Initial wound healing involves the synthesis of type III collagen, which is characteristic for immature connective tissue. As wound healing progresses, type III collagen, which is the main component of the granulation tissue, is replaced by type I collagen. Type I collagen is the main and most common type present in dermal tissue and is responsible for the tensile strength of the tissue.[14] Our results suggest that tamanu oil can aid in early stages of the healing process by increasing the expression of type III collagen by human fibroblasts. In wound healing and inflammatory processes, the development of scar remodeling is important in order to obtain a sufficiently strong substitute tissue that is resistant to mechanical stimuli.[15,16] The synthesis of collagen proteins is a very complex process, which is dependent on the level of the natural antioxidant, vitamin C.[17,18] Thus, the expression of main antioxidant enzyme MnSOD was evaluated. The MnSOD protein content was found to increase with increasing pulsed electric field intensity combined with tamanu oil use, in comparison to control non-treated cells.

Conclusions

It has been shown that the *C. inophyllum* extract stimulates the proliferation of established and primary fibroblasts to a different degree. The use of tamanu oil at the concentrations indicated by this study suggests that the stimulated proliferation process could be utilized as an anti-inflammatory, analgesic and antiseptic agent in the healing process (increased expression of collagen type III) of vaginal infections. Moreover, tamanu oil is known for its strong antimicrobial properties. The obtained results might be the basis for the future development of protocols involving the application of the EP method combined with the oil extract from *C. inophyllum* in preclinical and clinical treatment.

References

1. Abbaspoor Z, Rabee Z, Najjar S. Efficacy and safety of oral tinidazole and metronidazole in treatment of bacterial vaginosis: A randomized control trial. *Int J Clin Pharmacol Res.* 2014;4(2):2277–3312.
2. Edwards L. The diagnosis and treatment of infections vaginitis. *Dermatol Ther.* 2004;17(1):102–110.
3. Mitchell H. ABC of sexually transmitted infections. Vaginal discharge: Causes, diagnosis and treatment. *BMJ.* 2004;29(7451):1306–1308.
4. Edwards L. Dermatologic causes of vaginitis: A clinical review. *Dermatol Clin.* 2010;28(4):727–735.
5. Antachopoulos C, Roilides E. Cytokines and fungal infections. *Br J Haematol.* 2005;129(5):583–596.
6. Choromańska A, Kulbacka J, Rembiałkowska N, et al. Anticancer properties of low molecular weight oat beta glucan: An in vitro study. *Int J Biol Macromol.* 2015;80:23–28.
7. Rahar S, Swami G, Nagpal N, Nagpal MA. Preparation, characterization and biological properties of b-glucans. *J Adv Pharm Technol Res.* 2011;2(2):94–103.
8. Drag-Zalesińska M, Wysocka T, Borska S, et al. The new esters derivatives of betulin and betulinic acid in epidermoid squamous carcinoma treatment: In vitro studies. *Biomed Pharmacother.* 2015;72:91–97.
9. Bautista CT, Wurapa E, Sateren WB, Morris S, Hollingsworth B, Sanchez JL. Bacterial vaginosis: A synthesis of the literature on etiology, prevalence, risk factors, and relationship with chlamydia and gonorrhea infections. *Mil Med Res.* 2016;3:4.
10. Saczko J, Kamińska I, Kotulska M, et al. Combination of therapy with 5-fluorouracil and cisplatin with electroporation in human ovarian carcinoma model in vitro. *Biomed Pharmacother.* 2014;68(5):573–580.
11. Hasan AM. Review on: Vaginitis. A problem to be solved! *J Pharm Chem Biol Sci.* 2016;4(1):104–110.
12. Léguillier T, Lecsö-Bornet M, Lémus C, et al. The wound healing and antibacterial activity of five ethnomedical *Calophyllum inophyllum* oils: An alternative therapeutic strategy to treat infected wounds. *PLoS One.* 2015;10(9):e0138602.
13. Smith JD, Garber GE. Trichomonas vaginalis infection induces vaginal CD4 cell infiltration in a mouse model: A vaccine strategy to reduce vaginal infection and HIV transmission. *J Infect Dis.* 2015; 212(2):285–293.
14. Darby IA, Laverdet B, Bonté F, Desmoulière A. Fibroblast and myofibroblast in wound healing. *Clin Cosmet Investig Dermatol.* 2014;7:301–311.
15. Osiak K. Przerostowe blizny, bliznowce i przykurcze bliznowate. *Post Nauk Med.* 2005;18:17–23.
16. Xue M, Jackson CJ. Extracellular matrix reorganization during wound healing and its impact on abnormal scarring. *Adv Wound Care (New Rochelle).* 2016;4(3):130–136.
17. Grosso G, Bei R, Mistretta A, et al. Effects of vitamin C on health: A review of evidence. *Front Biosci (Landmark Ed).* 2013;18:1017–1029.
18. van Kessel K, Assefi N, Marrazzo J, Eckert L. Common complementary and alternative therapies for yeast vaginitis and bacterial vaginosis: A systematic review. *Obst Gynecol Surv.* 2003;58(5):351–358.