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Lecithin-based nanoemulsions of traditional herbal wound healing agents and their effect on human skin cells

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ABSTRACT

In previous studies, lecithin-based nanoemulsions (NEs) have been shown to be skin friendly drug carrier systems. Due to their nontoxic properties, NEs might also be suitable as wound healing agents. Hence, different O/W NEs based on lecithin Lipoid® S 75 and plant oils or medium chain triglycerides were produced and characterised. Two lipophilic natural wound healing agents, a betulin-enriched extract from birch bark (BET) and a purified spruce balm (PSB), were successfully incorporated and their effects on primary human skin cells were studied *in vitro*. MTT, BrdU and scratch assays uncovered the positive influence of the drug-loaded NEs on cell viability, proliferation and potential wound closure. Compared to control formulations, the NEs loaded with either BET or PSB led to higher cell viability rates of fibroblasts and keratinocytes. Higher proliferative activity of keratinocytes and fibroblasts was observed after the treatment, which is a prerequisite for wound closure. Indeed, in scratch assays NEs with PSB and notably BET showed significantly ameliorated wound closure rates than the negative control (unloaded NEs) and the positive control (NEs with dexpanthenol). Our findings suggest that BET and PSB are outstanding wound healing drugs and their incorporation into lecithin-based NEs may represent a valid strategy for wound care.

1. Introduction

The skin, the largest organ of the human body, represents a protective barrier not only against the loss of water and ingress of foreign and possible noxious material, but also against pathogens and microorganisms [1,2]. Loss of integrity of this barrier can lead to painful and possibly dangerous conditions, such as skin ulcers or chronic wounds. Upon wounding, certain processes are initiated to eventually restore the integrity of the damaged area. These processes can be divided into different phases: an inflammatory phase, followed by a granulation phase with synthesis of new tissue and epithelial wound closure, and finally a scar-remodelling phase [3–7]. During the whole repair process, different types of cells work together and interact extensively with each other. Of particular importance are fibroblasts and keratinocytes due to their prominent role in the granulation and re-epithelialization phase, respectively [5]. Accordingly, when studying potential wound healing formulations or drugs, tests with both cell types are of particular interest. However, most wound healing experiments so far have been carried out with keratinocytes, but not with the equally or even more important dermal fibroblasts [8]. Fibroblasts are critical in all wound healing phases, in particular when depositing extracellular matrix components, contracting the wound and remodelling of new extracellular matrix [9]. Hence, they play a key role in the wound healing process and should be considered in wound healing studies. Another shortcoming of previously published data is the use of immortalised cell lines instead of primary skin cells. Though a good first orientation in preliminary studies, the results obtained with immortalised cell lines may differ remarkably from the *in vivo* situation [10–12].

Abbreviations: BET, betulin-enriched extract; BrdU, 5-bromo-2′-deoxyuridine; DEX, dexpanthenol; ELISA, enzyme-linked immunosorbent assay; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NE, nanoemulsion; PBS, Phosphate-buffered saline; PDI, polydispersity index; PSB, purified spruce balm.

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After an injury of the skin, the main goal is to close the wound rapidly and thus reduce risk of infection [13]. For centuries, people have used plants, their parts, extracts and preparations thereof in skin treatment, especially for wound healing purposes [3,14]. Birch (*Betula species*), in particular its bark, has been used as a natural remedy for skin diseases and wound care for centuries [15]. Betulin, the main active compound extracted from the outer bark of birch, is a triterpene that exhibits a variety of pharmacological properties like anti-inflammatory, antiviral and wound-healing effects [16]. In recent studies, the wound healing properties of betulin as well as of a heptane extract of birch bark have been clinically confirmed in treatment of necrotic and burned skin. In other studies, accelerated re-epithelialization after invasive procedures in humans was found [17–19].

Another traditionally used wound healing substance is the balm of Norway spruce (*Picea abies*). Scandinavian ointments prepared from Norway spruce balm have been used for centuries to treat acutely and chronically infected wounds due to its antimicrobial features [20,21]. Various clinical trials have confirmed its empirical use; however, the active components, their mode of action, and the exact composition were unknown until recently [22]. Goels et al. subjected Norway spruce balm to fractionated extraction and purification. Hydroxycinnamic acids, the lignan pinoresinol, four hydroxylated derivatives of dehydroabietic acid, and dehydroabietic acid were isolated and their structures analysed. Further, these compounds were tested for increased re-epithelialization of cell-free areas in a keratinocyte monolayer. The main compounds of Norway spruce balm, lignans and diterpene resin acids, accelerated wound closure by increasing migration and proliferation of keratinocytes *in vitro* [22].

Since damaged skin is a very sensitive target for application of dermal preparations, there is a clear need for highly skin compatible compounds when preparing wound-healing formulations. Phospholipid-based surfactants are therefore among the primary options for this task. Lecithin-based surfactant mixtures are known for their high biocompatibility and great emulsifying properties due to their amphiphilic nature and offer a broad range of applications in pharmaceutical technology [23–25]. Traditional surfactants that were formerly used as standard in cosmetic and pharmaceutical preparations, such as the anionic sodium dodecyl sulfate or sodium laureth sulfate, have fallen into disrepute due to their aggressiveness towards the skin [26–28]. Hence, there are expectations that lecithins could replace skin damaging emulsifiers. They are inherently eco-friendly, as they are mixtures of naturally occurring phosphatidylcholines and have a reputation of being more skin friendly than conventional surfactants. Our studies have recently confirmed this *in vitro* [29], as well as *in vivo* [30]. From a technological viewpoint, a suitable vehicle for wound treatment would be lecithin-based nanoemulsions (NEs). These fluid oil-in-water systems exhibit small droplet sizes and high storage stability, which makes them excellent vehicles for active pharmaceutical ingredients [31]. Since both betulin-enriched extract (BET) and purified spruce balm (PSB) are of lipophilic nature, NEs offer the option of incorporation into the oil phase.

Thus, the aim of this study was to develop NEs based on lecithin for incorporation of BET and PSB as skin-friendly wound healing formulations to be evaluated on human primary skin cells *in vitro*. The NEs' effect on cell viability and proliferation was evaluated by MTT and BrdU assays, respectively, using primary human keratinocytes and fibroblasts. Furthermore, their wound healing potential was evaluated by means of scratch assays using primary human fibroblasts. Scratch assays are a simple and well-established method to measure cell migration *in vitro*. After creating a ''scratch'' in a cell monolayer, images at the beginning and at regular intervals during cell migration and culture duration are recorded and compared to quantify the cell migration rate [32]. The results were compared with drug-free NEs as negative control and NEs containing the prominent wound healing substance dexpanthenol (DEX) as basis for comparison.

2. Materials and methods

2.1. Materials

Soybean lecithin Lipoid® S75 (soybean phospholipids with phosphatidylcholine content of 70%) was kindly provided by Lipoid GmbH (Ludwigshafen, Germany). Dexpanthenol, jojoba oil, medium-chain triglycerides and sunflower oil were purchased from Herba Chemosan Apotheker-AG (Vienna, Austria). Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum and Penicillin-Streptomycin (10,000 U/mL) were obtained from Gibco by Life TechnologiesTM (Carlsbad, USA). Keratinocyte Growth Medium 2 (KGM-2) was purchased from PromoCell GmbH (Heidelberg, Germany) and from Lonza (Basel, Switzerland). Cell proliferation assay (EZ4U) was obtained from Biomedica Medizinprodukte GmbH & Co KG (Vienna, Austria). BrdU immunosorbent assay was purchased from Roche Diagnostics (Indianapolis, IN, USA). 2-well silicone inserts for scratch assay were obtained from ibidi GmbH (Gräfelfing, Germany). All other chemicals were of analytical reagent grade and used without further purification.

2.2. Plant material

Birch bark (*Betula pendula Roth., Betulaceae*) was supplied by Kottas Pharma GmbH (BN. KLA30556). A betulin-enriched birch bark extract was prepared suspending 10.0 g of birch bark in 300 mL ethanol 96%. 20% aqueous KOH solution was heated to 80 °C in a water bath for 8 h. The hot mixture was filtered and left for precipitation overnight in the fridge. The supernatant was removed and 544.6 mg of the precipitate corresponding to BET were gained [33].

Spruce balm from *Picea abies* was identified according to the monograph in the Austrian Pharmacopoeia and collected in a forest near Sauerfeld, Lungau, Austria (47°07′5.0″N; 13°52′50.0″E) by harvesting the balm from the tree trunk with a piece of tree bark. The gained balm was stored at –20 °C ahead of further processing. Following the traditional way of preparation, spruce balm was subjected to a boiling step before extraction [34]. Deionised water was heated up to approximately 90 °C in a metal vessel. A metal sieve containing the balm was covered with a linen cloth and placed on a wooden spoon across the vessel so that the balm dangled freely into the boiling water. The high temperature decreased the viscosity of the spruce balm and provoked single drops to pass through the linen tissue and sieve into the water. After temperature equalisation, PSB, which had gathered at the bottom of the vessel, was gained by decantation. Its yield amounted to 431 g [22]. The yield of this procedure was used for analysis and preparation of the NEs.

2.3. Analysis of betulin-enriched extract via HPLC

For HPLC analysis a Shimadzu instrumentation (Shimadzu Degasser DGU-20A 5, Shimadzu Auto Sampler SIL-20AC HAT, Shimadzu Communications Bus Module CBM-20A, Shimadzu Liquid Chromatograph LC-20AD, Shimadzu Column Oven CTO-20AC, Shimadzu Diode Array Detector SPD-M20A, Shimadzu ELSD-LT, SHIMADZU Lab solutions Software) was used. An RP18 column (LiChrospher 100 RP18e 5 µm, 250 mm × 4 mm) served as stationary phase. Elution of the analytes was realised using double distilled water + 0.1% formic acid (solvent A) and acetonitrile + 0.1% formic acid as mobile phase (solvent B). The isocratic elution started at 85% solvent B for 30 min at a flow rate of 1 mL/min. Analysis via HPLC-ELSD with a retention time of 20.3 min yielded in 69.2% betulin in the enriched extract, see Supplementary Fig. 1.

2.4. Analysis of PSB via GC-FID

For the analysis via GC-FID the gas chromatograph Perkin Elmer XL Autosystem with a DB-1701 column (14%-Cyanopropyl-phenyl-methylpolysiloxane, length: 60 m, inner diameter: 0.25 mm, layer thickness: 0.25 µm) and nitrogen as mobile phase (flow: 2 mL/min) were

employed. 1 µl of sample in a concentration of 2.5 mg/mL for the quantitation was injected into the system via an autosampler. Separation took place in splitless mode using a temperature gradient starting at 100 °C (hold for 1 min) and elevating to 200 °C with 15 °C/min and then with 2 °C/min to 280 °C (hold for 10 min). For detection a flame ionization detector was used.

The analysis of the samples was preceded by a derivatization step to enhance volatility on the resin acids. As derivatization reagent methyl-8 reagent (N,N-dimethylformamide dimethyl acetal; purity ≥ 98%; BN: 079215805; Carl Roth) was employed to methylate the carboxylic acid function of the resin acids and thereby increase their volatility to an extend that is applicable in gas chromatography. The dissolved samples were migrated to a vial, 300 µg methyl palmitate, dissolved in ethanol, were added as internal standard and the solvent was removed with pressurized air and dried in the desiccator. 200 µl of the derivatization agent were added and heated to 85 °C for 30 min. After a cooling phase of approx. 30 min 200 µl of pyridine (pyridine dried p.a.; BN: 1792463; Merck) were added and analysed via GC-FID. The chromatogram of PSB can be found in Supplementary Fig. 2-X.

2.5. Preparation of oil-in-water nanoemulsions

For the preparation of the NEs, we chose three common oils: jojoba oil (jojo), medium-chain triglycerides (mct) and sunflower oil (sun). 5% (w/w) of the surfactant was dissolved and stirred for 1 h at 60 °C. After 1 h, the oily phase containing 0.1% (w/w) BET or PSB, was added slowly under vigorous stirring. Subsequently, the formulations were treated with a high-pressure homogenizer (Avestin Emulsiflex C3, Avestin, Canada) for 10 cycles at 1000 bar. The composition of the prepared NEs can be found in Table 1.

2.6. Physical characterisation

Droplet sizes, polydispersity index (PDI) values and zeta potential of all prepared formulations were analysed by dynamic light scattering technology and laser Doppler electrophoresis using a Zetasizer Nano ZS (Malvern Instruments, Malvern, UK). Samples were diluted with freshly distilled water 1:100 (v/v) containing sodium chloride (0.01 mmol) and analysed at 25 °C. Dynamic light scattering and laser Doppler electrophoresis require appropriate dilution of samples for correct measuring to avoid e.g. multiple scattering. Both distilled water and electrolyte solutions have been used for this task. Dilution with pure distilled water however might lead to fluctuating conductivity. Therefore, electrolyte solutions of distilled water and NaCl or KCl are frequently employed to ensure constant conductivity [35–37].

2.7. Cell culture studies employing primary human skin cells

2.7.1. Ethics statement, isolation and cultivation of keratinocytes and fibroblasts

Skin from anonymous healthy female and male donors (age range: 25–62) was obtained during plastic surgery procedures (abdomen, upper arm). The study was approved by the ethics committee of the Medical University of Vienna and conducted in accordance with the principles of the Declaration of Helsinki. Written informed consent was obtained from the participants (vote number 1149/2011). The isolation and cultivation of primary skin cells were performed according to a previously described method [29,38,39]. For cytotoxicity assays on keratinocytes and fibroblasts, cells from different donors (n = 4–5) were used, and the results averaged.

Table 1
Composition of NEs* in % (w/w). BET: betulin-enriched extract; PSB: purified spruce balm.

Formulation	S75-jojo	S75-mct	S75-sun
Surfactant	5% Lipoid® S75	5% Lipoid® S75	5% Lipoid® S75
Oil phase	10% jojoba oil	10% medium chain triglycerides	10% sunflower seed oil
Water	ad 100%	ad 100%	ad 100%
BET or PSB	0.1%	0.1%	0.1%

* Blank and drug-loaded formulations were produced.

2.7.2. Assessment of cell viability and proliferation

For assessing cell viability after the treatment with different NEs, we used two different cell viability assays. The EZ4U cell proliferation assay (Biomedica, Vienna, Austria) is a modification of the MTT test (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) that scans for mitochondrial damage in cells and therefore correlates with cell viability [40]. The BrdU assay admits a suitable tool for the quantitative measurement of DNA replication, measuring the amount of the pyrimidine analogon BrdU being incorporated into DNA instead of thymidine [41]. Cellular incorporation of BrdU can be detected by anti-BrdU-specific antibodies following membrane permeabilisation via an enzyme-linked immunosorbent assay (ELISA).

For both assays, primary cells were suspended in colourless supplemented culture medium at a density of 10^4 cells per well for fibroblasts and 1.5×10^4 cells per well for keratinocytes and seeded on 96-well flat-bottom plates (200 µl/well) (Greiner bio-One, Solingen, Germany). After incubation for 24 h at 37 °C and 5% CO_2 air atmosphere, NEs were added to the culture medium in different dilutions. Both assays were performed following the manufacturer's protocol [29,39].

For the EZ4U assay, the NEs were removed from the culture plates after 2 h of incubation before adding colourless medium containing the EZ4U-substrate. Colorimetric staining of the cells and the supernatant by the formazane formed was evaluated using a multiwell plate reader at 450 nm (Tecan™ infinite 200, Tecan Ltd., Maennedorf, Switzerland), with a reference wavelength at 620 nm. High absorbance indicated a high number of mitochondrial active cells.

For the BrdU assay, the NEs were removed and BrdU was added to the cells following re-incubation for 2 h. During this labelling period, thymidine is replaced by BrdU in the DNA of proliferating cells. After removing the culture medium, the cells were fixed, and the DNA was denatured in one step by adding FixDenat. The added anti-BrdU antibody binds to the BrdU incorporated in newly synthesised cellular DNA. Finally, the immune complexes are detected by the subsequent substrate reaction. The reaction product is quantified by measuring the absorbance at 370 nm with a reference wavelength set to 492 nm using a scanning multiwell spectrophotometer (ELISA reader). The developed colour and thereby the absorbance values directly correlate to the amount of DNA synthesis and hereby to the number of proliferating cells.

2.7.3. In vitro wound healing assays

To monitor the effect of the NEs on the wound closure on primary fibroblasts, wound healing assays were performed with modifications based on the protocol of Liang et al. [19]. For this purpose, 2-well silicone inserts from ibidi GmbH (Germany) were placed in the wells of 24 well plates. This guarantees a constant and reproducible cell-free gap (≙ artificial wound) of 500 µm. Afterwards, fibroblasts were seeded with the density of 2×10^5 cells per well and allowed to form a confluent monolayer around the silicone insert for the next 24 h (37 °C, 5% CO_2). After 24 h the inserts were removed and new medium containing NEs dilutions (1:1000) were added (t = 0) and placed in the incubator. The blank, unloaded NEs served as negative control, NEs with incorporated DEX served as a positive control. Photos of the cell-free gaps at the starting time (t = 0) and after 48 h (t = 48) were taken. The cell-free gap was analysed by the image processing programme ImageJ combined with the ImageJ macro wound healing tool [42]. For calculating the wound closure in % after 48 h, we used the following equation:

$$wound\ closure\ [\%] = 100 - \left(\frac{cell\ free\ gap\ area\ t\ =\ 48}{cell\ free\ gap\ area\ t\ =\ 0} \times 100\right)$$

2.8. Statistical analysis

Results are expressed as mean values ± standard deviation (SD). Data were analysed using the JASP 0.9.0.0 software using the Shapiro-Wilk test as test of normality and one-way ANOVA + post-hoc Tukey test or the Student's *t*-test with $p < 0.05$ as minimum level of significance.

3. Results and discussion

3.1. Nanoemulsion characterisation

The incorporation of the two lipophilic active ingredients led to fluid white NEs, with their physicochemical properties shown in Table 2-A and Table 2-B and . Mean droplet sizes were in the range of 153 to 245 nm. In all cases, the incorporation of one of the two lipophilic substances led to a decrease in droplet size. These difference reached statistical significance in case of S75-jojo-BET and S75-sun-BET ($p \leq 0.01$). PDI values ranged from 0.17 to 0.24. Zeta potentials ranged from −67.58 to −77.13 mV. Thus, satisfying homogeneity and good electrochemical stability due to repulsion of droplets was assumed. Drug-free control NEs have already been extensively characterised before; data can be found in our recently published work [39]. Lipophilic drugs may potentially alter the properties of the interfacial film through inserting themselves between the lipophilic tails of phospholipids [43], usually inducing a slight increase in particle sizes [36,43]. The effect may however also be less pronounced or negligible if the lipophilic actives are dissolved in the oil phase without affecting the interfacial film [39]. The observed effect of significantly decreased particle size after incorporation of BET might be caused by the triterpene structure of the drug. This could influence membrane fluidity and thus affect the physicochemical properties of the produced NEs [44,45].

3.2. Effects of NEs on human skin cell viability and proliferation

Next, we monitored the cytotoxic effects of drug-loaded NEs in different dilutions (cytotoxic profile of the various NEs) and determined the optimal dilution to perform scratch assays to investigate wound healing. It was envisioned to find the dilution that would yield a cell viability rate of 100% for every NE, so that none of the formulations would have a "head start" and thus make interpretation of the results more challenging. First, EZ4U assays were performed; a dilution of 1:200 was tested (Fig. 1). The results using primary fibroblasts are shown in Fig. 1-A. With all three oils, the cytotoxic tendency appeared similar: the unloaded NEs yielded the lowest viability rates of around 70%, followed by the NEs with BET as the active ingredient with 70 to 75%, and with NEs with PSB yielding the highest viability rates ranging from 83 to 91%. The viability rates of cells treated with S75-mct-PSB when compared to the cells treated with S75-mct were significantly higher ($p \leq 0.05$). With the EZ4U assay on primary keratinocytes (Fig. 1-B), we yielded similar results for all NEs, with keratinocyte viability rates from 69 to 77%, with no statistically significant differences caused by BET or PSB. However, small trends towards higher viability rates for cells treated with the loaded formulations could be seen.

As the 1:200 dilution still led to lower viability rates for the unloaded and BET containing NEs, we next tested a higher dilution (1:400, Fig. 2). The viability rates of fibroblasts (Fig. 2-A) treated with the PSB

Table 2B
Droplet size, PDI and zeta potential of blank S75-based NEs vs. NEs loaded with purified spruce balm (-PSB).

Formulation	S75 -jojo	S75 -jojo-PSB	S75 -mct	S75 -mct-PSB	S75 -sun	S75 -sun-PSB
Droplet size (nm) (±SD); week 0	234.20 (20.40)	231.34 (7.58)	169.20 (2.80)	168.31 (7.63)	245.20 (9.70)	232.40 (7.16)
PDI (±SD)	0.23 (0.01)	0.23 (0.00)	0.17 (0.01)	0.19 (0.01)	0.22 (0.02)	0.24 (0.01)
Zeta potential (mV) (±SD)	−73.83 (4.90)	−68.60 (2.62)	−81.61 (1.88)	−76.43 (4.92)	−68.53 (3.92)	−67.76 (2.41)

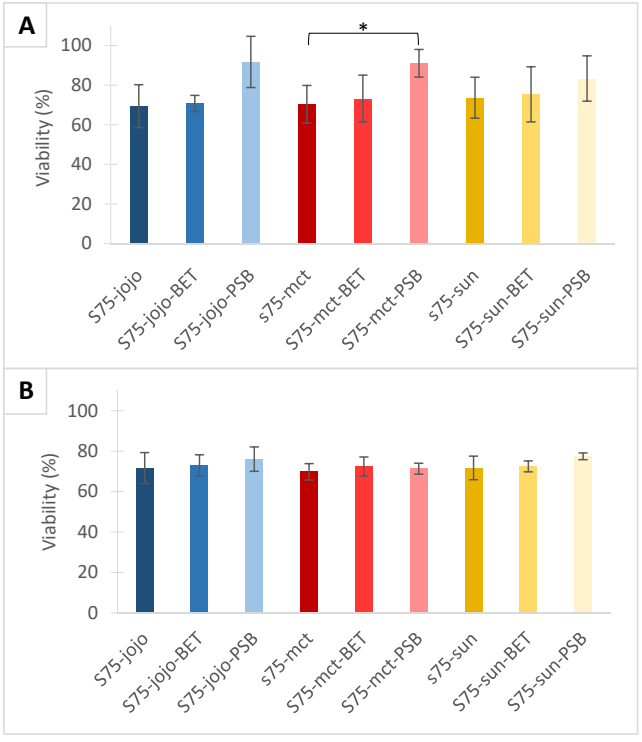

Fig. 1. Cell viability of human primary fibroblasts (A) and human primary keratinocytes (B) after 2 h of incubation with different NEs (dilution of 1:200). Data are expressed as % viability, ±SD, n = 4 with 12 parallel experiments per formulation. Statistical differences are marked with asterisks (*$p < 0.05$) and were tested as one-way ANOVA + post-hoc Tukey test with $p < 0.05$ as minimum level of significance. BET: betulin-enriched extract; PSB: purified spruce balm.

Table 2A
Droplet size, PDI and zeta potential of blank S75-based NEs vs. NEs loaded with betulin enriched extract (-BET). *

Formulation	S75 -jojo	S75 -jojo-BET	S75 -mct	S75 -mct-BET	S75 -sun	S75 -sun-BET
Droplet size (nm) (±SD); week 0	234.20 (20.40)	189.88** (8.33)	169.20 (2.80)	153.31 (11.70)	245.20 (9.70)	214.63** (7.48)
PDI (±SD)	0.23 (0.01)	0.19*** (0.01)	0.17 (0.01)	0.19* (0.01)	0.22 (0.02)	0.23 (0.01)
Zeta potential (mV) (±SD)	−73.83 (4.90)	−67.58 (3.18)	−81.61 (1.88)	−77.13* (2.61)	−68.53 (3.92)	−70.90 (4.29)

* Statistically significant differences to blank formulations are marked with asterisks (*$p < 0.05$ **$p < 0.01$ ***$p < 0.001$) and were analysed using Student's *t*-test.

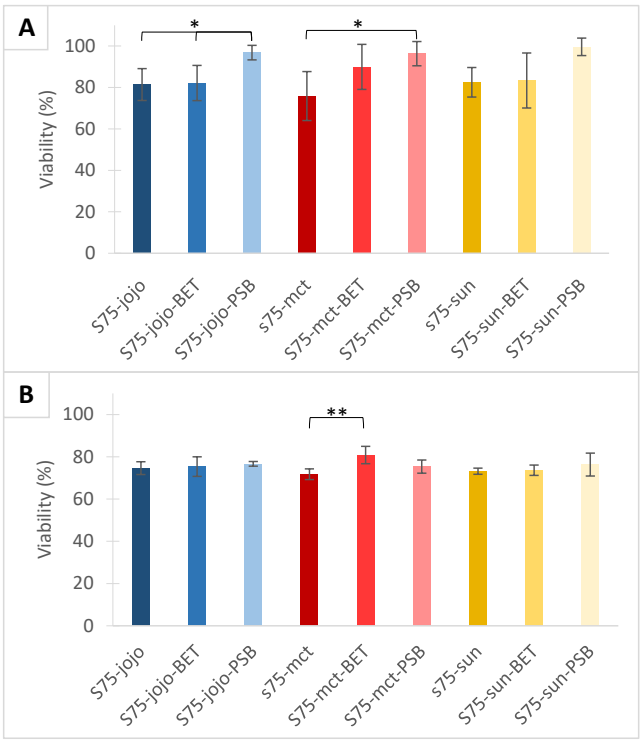

Fig. 2. Cell viability of human primary fibroblasts (A) and primary human keratinocytes (B) after 2 hours of incubation with NEs (dilution of 1:400). Data are expressed as % viability, ±SD, n=4 with 12 parallel experiments per formulation. Statistical differences are marked with asterisks (*p < 0.05, **p < 0.01) and were tested as one-way ANOVA + post-hoc Tukey test with p < 0.05 as minimum level of significance. BET: betulin-enriched extract; PSB: purified spruce balm.

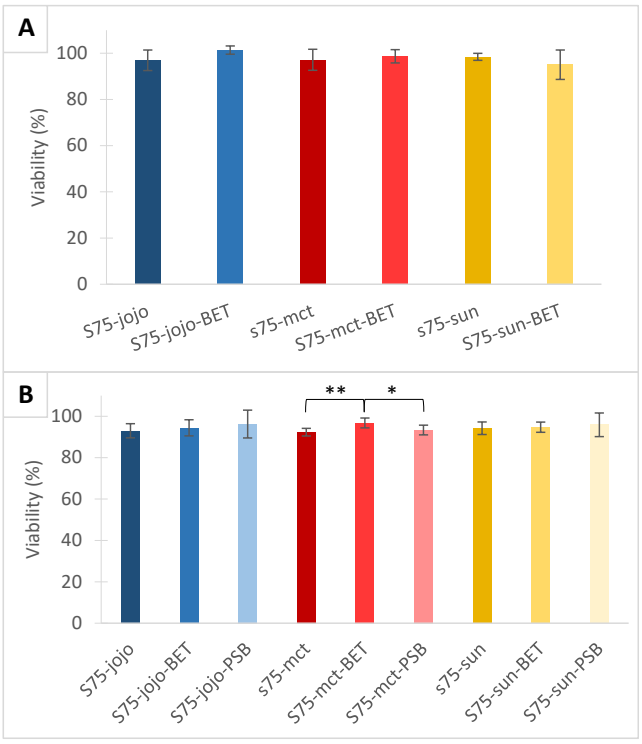

Fig. 3. Cell viability of human primary fibroblasts (A) and human primary keratinocytes (B) after 2 h of incubation with NEs (dilution of 1:1000). Data are expressed as % viability, ±SD, n = 4 with 12 parallel experiments per formulation. Statistical differences are marked with asterisks (*p < 0.05, **p < 0.01) and were tested as one-way ANOVA + post-hoc Tukey test with p < 0.05 as minimum level of significance. BET: betulin-enriched extract; PSB: purified spruce balm.

containing formulations reached around 100%, whereas the unloaded NEs and BET-NEs still showed significantly lower viability rates in some cases when compared to the PSB-NEs, more precisely in the cases of S75-jojo and S75-jojo-BET compared to S75-jojo-PSB ($p \leq 0.05$) and S75-mct versus S75-mct-PSB ($p \leq 0.05$). As the formulations with PSB yielded around 100%, we refrained from further diluting these NEs and concentrated on the unloaded NEs and NEs containing BET. Regarding the keratinocyte viability rates (Fig. 2-B), two observations stood out: first, the viability rates seemed to increase slower with dilution when compared to the fibroblasts; keratinocytes yielded only 71 to 80% viability at a dilution of 1:400 versus 69 to 77% at a dilution of 1:200. Second, drug incorporation into the NEs seemed to have a minor impact on the viability rates of keratinocytes when compared to the fibroblasts, although again a slight trend towards higher viability of drug loaded formulations was perceptible. In the case of S75-mct versus S75-mct-BET this difference was statistically significant (**p < 0.01).

Finally, a dilution of 1:1000 was tested (Fig. 3). Fig. 3-A shows experiments on fibroblasts of the remaining NEs. Again, also with this dilution cell viability rates about 100% were reached, irrespective whether BET was included or not. Fig. 3-B shows experiments on keratinocytes with the dilution 1:1000. Keratinocyte viability reached 92 to 97% after treatment with the indicated NEs, and with statistically significant differences in case of S75-mct-BET versus S75-mct (**p < 0.01) and S75-mct-BET versus S75-mct-PSB (*p < 0.05). Considering these high viability rates, especially in the primary fibroblasts, we identified the dilution 1:1000 as optimal for the planned *in vitro* wound healing assays.

Having completed the EZ4U assays, BrdU assays were performed. The MTT assay only scans for mitochondrial activity in cells, but does not provide information about the cells' ability to proliferate, which is essential when developing wound healing formulations. Therefore, we also employed the BrdU assay, which might reveal potential effects of BET and PSB on cell proliferation. With the MTT results of the 1:200 dilution experiments in mind, we used this dilution for the BrdU tests as we expected differences not only in mitochondrial damage, but also in the proliferative effect of loaded versus unloaded NEs.

The results of the BrdU assays are given in Fig. 4, again starting with fibroblasts (Fig. 4-A). The unloaded formulations led to lower proliferative ability in all cases (56–64%) when compared to the loaded NEs (66–70%), however these differences were not statistically significant. Neither a significant difference between the two active ingredients BET and PSB could be detected.

The results obtained with the BrdU assay on keratinocytes can be seen in Fig. 4-B. Again, a trend of slightly higher proliferative ability of cells treated with drug-loaded NEs can be observed, however, these differences are not statistically significant.

Betulin has been studied intensively, particularly its anti-HIV activity and cytotoxicity against a variety of tumor cell lines. Interestingly, this cytotoxicity has been reported to not affect human fibroblasts, indicating tumor specificity [46–48]. These findings suggest that the incorporation of Betulin should not contribute considerably to the cytotoxicity on dermal fibroblasts, which could be confirmed by means of the MTT and BrdU assays. In contrast, results indicated an even slightly increased viability, which could also be seen with the epidermal keratinocytes.

Regarding the cytotoxic effects of PSB on skin cells there is not much data available. Coșarcă et al. found no cytotoxic effects of spruce extracts on the HaCaT keratinocyte cell line, but increasing cell viability at all the tested concentrations tested, decreasing the dose even enhanced this effect [49]. This could also be seen in our experiments, including the experiments on primary fibroblasts.

Concerning the size of the nanoemulsion droplets and the

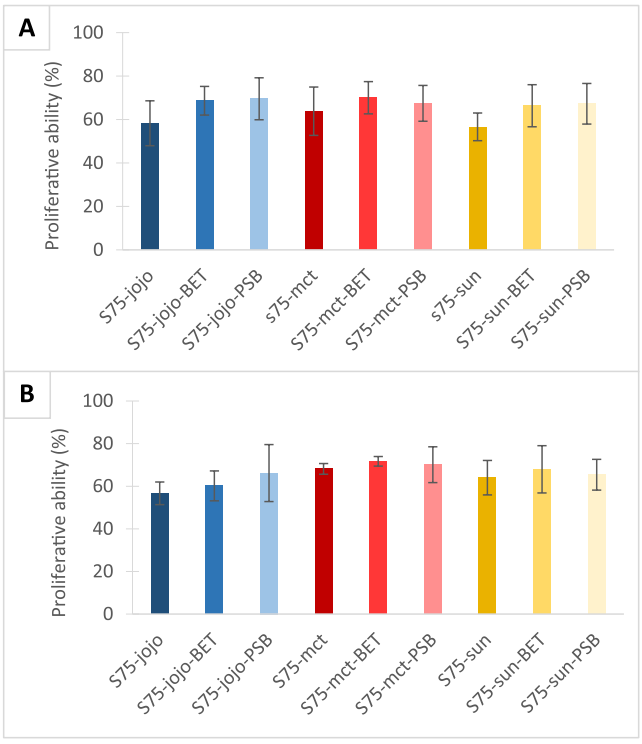

Fig. 4. Proliferative ability of human primary fibroblasts (A) and human primary keratinocytes (B) after 2 h of incubation with NEs (dilution of 1:200). Data are expressed as % proliferative ability, ±SD, n = 4 with 12 parallel experiments per formulation. BET: betulin-enriched extract; PSB: purified spruce balm.

concomitant properties, it can be assumed that a small droplet size alone will not automatically affect permeation/penetration of incorporated active agents from a soft carrier system; the crucial aspect seems to be the composition of the whole topically applied system [50].

3.3. Decisive effects of all NEs with an incorporated active ingredient on wound healing in vitro

Results of the scratch assays are given in Figs. 5 and 6. In general, when using primary human fibroblasts, NEs with incorporated active ingredients led to significantly smaller cell free gaps after 48 h in most cases (Fig. 6). An example of an *in vitro* scratch assay after treatment with the loaded and unloaded NE containing jojoba oil as the oil phase can be seen in Fig. 5. Surprisingly, NEs incorporated with the well-established wound healing drug DEX did not show superior wound healing properties when compared to NEs with BET or PSB as the active ingredient. Only in case of NEs with jojoba oil, the NE loaded with DEX was able to close the artificial wound significantly better than the NE loaded with PSB (Fig. 6-A, 6-B, and 6-C). In the case of the NEs with jojoba oil (Fig. 6-A), all three NEs with an active ingredient showed significantly smaller cell free gaps than the blank NE (S75-jojo wound closure: 61%). S75-jojo-BET had the highest wound closure with 83%, followed by S75-jojo-DEX with 77% and S75-jojo-PSB with 73%. In the case of the cells treated with NEs prepared with medium chain triglycerides (Fig. 6-B), the cells with blank NE and S75-mct-DEX yielded the same wound closure (71%), cells treated with S75-mct-PSB reached a wound closure of 73%, whereas fibroblasts treated with S75-mct-BET reached a wound closure of 84%, which was significantly higher than the wound closures obtained with the other NEs. Fibroblasts treated with NEs with sunflower oil (Fig. 6-C) as the oil phase showed a similar picture: S75-sun-BET reached the highest wound closure (91%), followed by S75-sun-PSB (82%) and S75-sun and S75-sun-DEX (76% and 75%, respectively).

Different acceleration rates of wound healing formulations with different oils have been examined in oleogels containing betulin by Steinbrenner et al. [47]. They found that some oils may significantly influence wound healing as vehicle compounds themselves. Additionally, the release of the active ingredient from the vehicle oil can either enhance or impair wound healing and a complex triangular interaction between wound, vehicle and betulin is assumed [47]. The influence of the oil on wound healing could explain why for example the unloaded NEs with sunflower oil and medium chain triglycerides were able to close the wound more efficiently than the unloaded NE with jojoba oil. In case of NE with sunflower oil versus NE with jojoba oil this difference in the acceleration of wound closure was statistically significant (**$p < 0.001$), see Supplementary Fig. 3-X.

Evidence for the wound healing effects of betulin has been gathered by several research groups over the years [8,17,47,51,52]. However, most wound healing experiments have been carried out with immortalised keratinocyte cell lines. Of note, primary dermal fibroblasts have not been included. Due to substantial interaction and cooperation between the two cell types during the wound healing process, it is conceivable that the following reported responses from keratinocytes

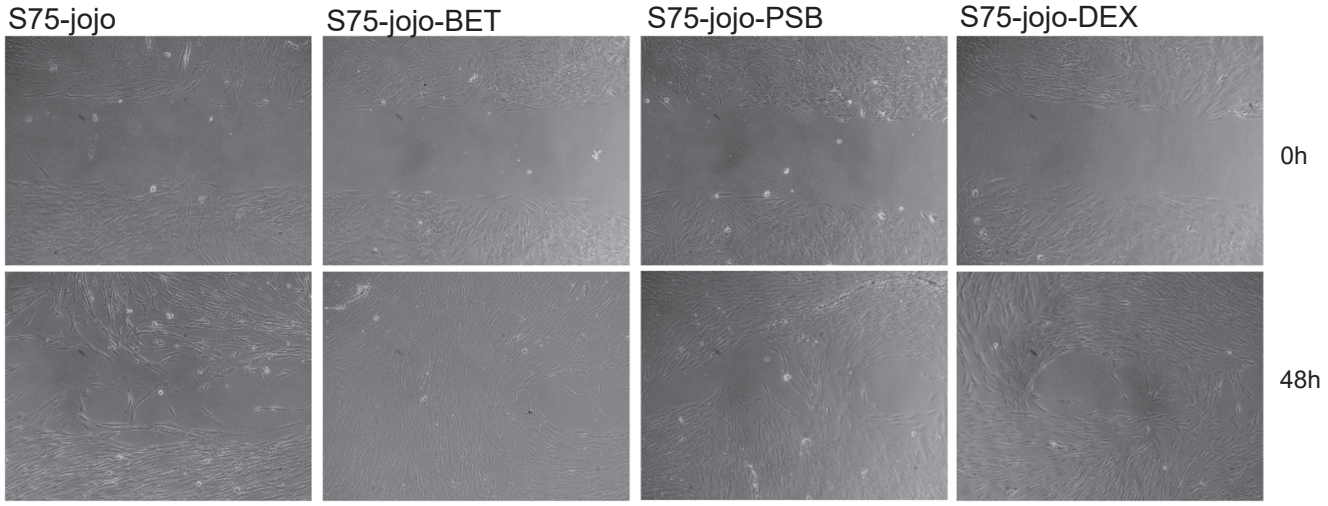

Fig. 5. In vitro scratch assay with unloaded and drug-loaded NE containing jojoba oil as the oil phase. Top row: photos at the beginning of the experiment (t = 0 h), bottom row: photos at the same spot after 48 h of treatment with the different nanoemulsions (t = 48 h). DEX: dexpanthenol; BET: betulin-enriched extract; PSB: purified spruce balm.

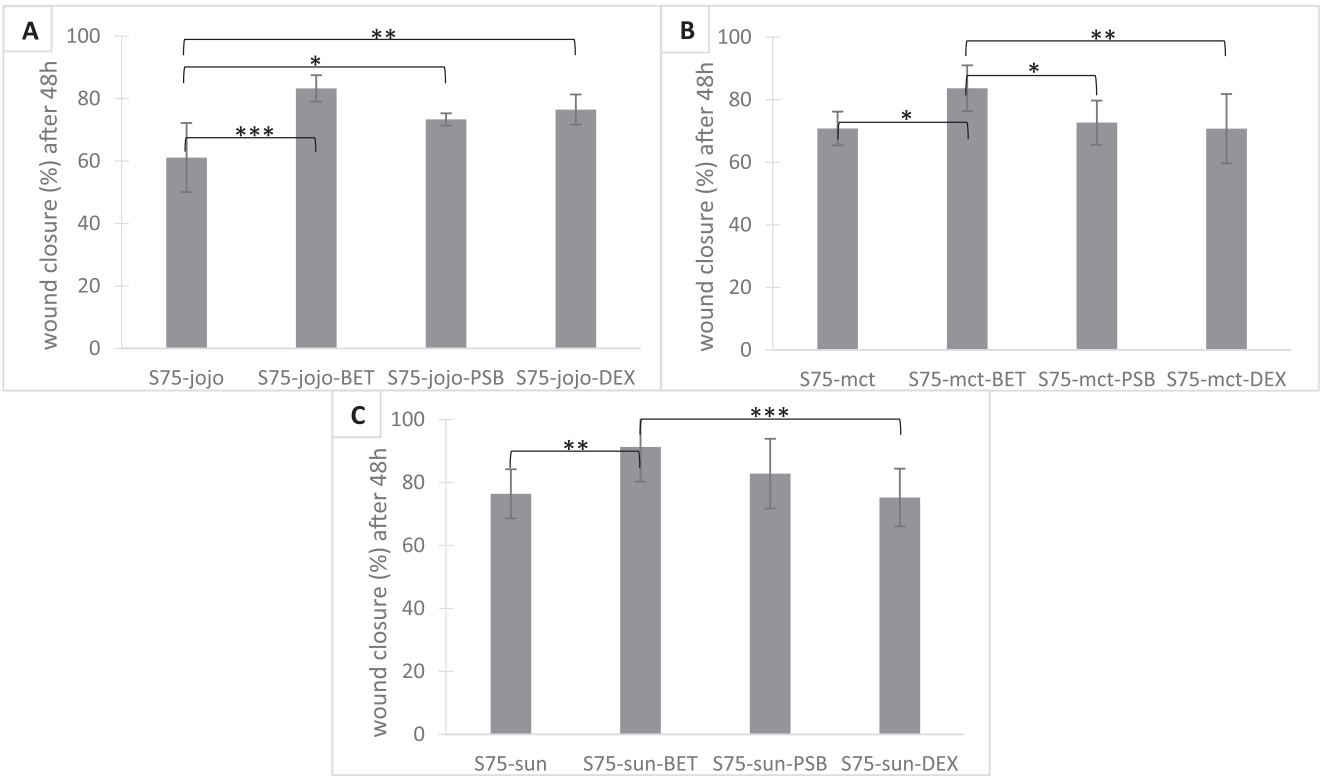

Fig. 6. Wound closures of fibroblast free gaps after 48 h treatment with different NEs: (A) NEs with jojoba oil, (B) NEs with medium chain triglycerides, (C) NEs with sunflower oil. Data are expressed as % wound closure, ±SD, n = min 6. Statistical differences are marked with asterisks (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$) and were tested as one-way ANOVA + post-hoc Tukey test with $p < 0.05$ as minimum level of significance. DEX: dexpanthenol; BET: betulin-enriched extract; PSB: purified spruce balm.

may be mirrored or supported in our scratch assays with dermal fibroblasts. Experiments using keratinocyte cell lines as reported in the literature showed that betulin affects wound healing at three stages. It is able to temporarily stimulate inflammation mediators such as cyclooxygenase-2 (COX-2), interleukin 6 (IL-6) or interleukin 8 (IL-8), which attract macrophages, phagocytes and granulocytes, initiating the cleaning of the wound [8,18]. In the second phase of healing, betulin influences the migration of skin cells via induction of IL-6 and signal transducer and activator of transcription 3 (STAT3). Another effect on keratinocytes is the increased formation of lamellipodia, filopodia and stress fibres. These structures are built when cells are stimulated to migrate until they come into contact with each other and accelerates re-epithelialisation [8,18]. In the third stage, when cells differentiate, mature and remodel a wound, betulin has been proven to stimulate differentiation markers like involucrin, keratin 10 and transglutaminase [8,53].

For the exact mode of action of the PSB on the quicker closing of wounds there is not as much data available as for betulin. However, several working groups have been working thoroughly on this issue. Especially the antimicrobial effect of PSB has been confirmed repeatedly [22,54,55]. Additionally, anti-inflammatory effects through the inhibition of interleukin 1-beta (IL-1β), matrix metalloproteinase-3 (MMP-3), and tumor necrosis factor alpha (TNF-α) has been reported [20]. These reports indicate that the wound healing effect of PSB mainly takes place in the inflammatory stage through neutralizing bacteria and inhibiting inflammation. Still, further research on the exact mode of action, especially in the later phases of the wound healing process, is of eminent interest and might provide important insight into the wound healing activity of PSB.

4. Conclusion

NEs containing BET or PSB showed higher skin cell viability at concentrations 1:200 and 1:400 in MTT tests than the control. BrdU studies demonstrated that skin cells treated with the loaded formulations also had a higher proliferative activity, which is essential for wound closure. NEs with PSB and especially BET displayed higher percentage wound closures in scratch assays than the negative control and the DEX-loaded positive control. These findings encourage further investigation into BET and PSB as potential wound healing drugs and their application in lecithin-based NEs may represent a valid strategy for wound care.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejpb.2021.11.004.

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