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A New Betulin Derivative Stimulates the Synthesis of Collagen in Human Fibroblasts Stronger than its Precursor

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Abstract. *Background/Aim: The exploration of substances that stimulate collagen synthesis and retard the aging process of the skin is an active field of current research. The natural environment and plants used in traditional medicine have been a source of such substances. The aim of this study was to compare the stimulatory effect of betulin (BE), betulinic acid (BA) and the new derivative – betulin ester with diaminobutyl acid (BE-Dab-NH_2) on collagen synthesis in human normal fibroblasts. Materials and Methods: Primary fibroblast cultures were obtained from the gums of a healthy patient. The effect of the above-mentioned compounds was assessed by Sircol collagen assay, immunocytochemistry, and proliferation test. Results: Fibroblasts cultured in the presence of BE-Dab-NH_2 produced 6.85 times more collagen than control cells, 7.85 times more than those cultured in the presence of BA and 6.31 times more than those cultured in the presence of BE. An intense immunocytochemical reaction for collagen type I and III was found in fibroblasts cultured in the presence of BE-Dab-NH_2. Conclusion: BE-Dab-NH_2 stimulates significantly more collagen synthesis in normal human fibroblasts than its precursor.*

Collagen is an extracellular matrix protein (ECM) synthesised mainly by fibroblasts. Currently, about 20 types of collagen are known, but collagen type I, II, III (skin, bones, teeth, cartilage, tendons, ligaments, fascia, cornea, sclera), and IV and VII (the area of basal membranes) are most common in our body. Type I collagen in the dermis forms thick bundles of irregular fibres, and collagen type III forms a fibril network. Collagen fibres type I and III combine with the remaining elements of the extracellular matrix – elastin fibres and proteoglycan complexes. Integration of ECM components and interaction of fibroblasts and ECM elements is possible thanks to adhesion molecules, fibronectin, entactin and laminin (1).

It is known that with age, gradual degradation of ECM components and a decrease in their synthesis by fibroblasts occurs. The process of skin aging is affected by hormonal changes, genetic conditions and extrinsic factors such as UV radiation (natural and from artificial sources), and smoking (2). Degradation and destruction of the collagen fibre architecture reduces the tensile strength of the skin, causing flaccidity and wrinkles (2).

The search for substances stimulating collagen synthesis and reducing the rate of skin aging processes is a constant direction in research. The natural environment, especially plants used in folk medicine, have been a source of such substances. Numerous studies have documented the action of triterpenes from *Centella asiatica* – madecassoside, madecassic and asiatic acid (3) on the synthesis of collagen. *Centella asiatica* (Asiatic pennywort) grows in Madagascar, and is traditionally used to heal wounds, to treat ulcers, and to improve the condition of the skin. Another natural product known to stimulate collagen synthesis as a co-factor of proline hydroxylase is ascorbic acid (4). Another triterpene, betulinic acid, which is a natural derivative of betulin has been shown to stimulate collagen synthesis (5). From the patent description, it appears that betulinic acid has a stronger stimulatory effect on collagen synthesis than the

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extract from *Centella asiatica*, and madecassoside and asiatic acid, active ingredients of this plant (5).

Betulin (BE) is a natural triterpene found in birch bark in an amount of about 10% - 30% and its only natural derivative is betulinic acid (BA), occurring in birch bark in an amount of about 1% (6). These compounds have a wide spectrum of properties including antioxidant, anti-inflammatory, antiviral, antibacterial and antineoplastic, being characterised by a lack of toxicity, both *in vitro* and *in vivo* (7). For this reason, for about 20 years they have been the focus of many studies as potential precursors of anticancer drugs (8). However, due to the hydrophobic nature of these substances (they show very low solubility in water), their use is very limited (9).

As a part of this study, new derivatives of betulin were designed and synthesised with more favourable biological properties than the initial compound. The method of synthesis of new betulin derivatives and the chemical structures of them has been described by Drag-Zalesinska *et al*. (10). Betulin-Dab-NH_2 derivative (betulin ester with L-2,4-diaminobutyl acid) showed a much greater ability to stimulate collagen synthesis in human fibroblasts than betulin and betulinic acid. This discovery has become the subject of patent PL 228855 B1 (11).

Materials and Methods

Betulin, betulinic acid and Betulin-Dab-NH_2. Betulin was extracted according to a previously published procedure (12) from the European white birch bark (*Betula pendula*, syn. *Betula verrucosa*) collected in the summer of 2012 in a forest near Wroclaw (Poland, Lower Silesia). The betulin derivative with the chemical name Betulin-Dab-NH_2 (betulin ester with L-2,4-diaminobutyl acid) was synthesised as part of the study of the National Science Centre project no. 0275/B/P01/2010/38. The synthesis method is described by Drag-Zalesinska *et al*. (10). Betulinic acid was purchased from Sigma company (Sigma, Poznan, Poland). Based on previous research (10), betulin (BE), betulinic acid (BA) and Betulin-Dab-NH_2 (BE-Dab-NH_2) were used in a concentration of 6 μM. The chemical structures of the compounds are shown in Figure 1.

Mass spectroscopy analysis. Mass spectrometry was conducted to confirm the molecular weights of synthesised Betulin-Dab-NH_2 compound with a high-resolution Waters LCT Premier XE mass spectrometer equipped with electrospray ionization (ESI) and time of flight (TOF) instruments.

Cell cultures. Primary fibroblast cultures were obtained from human gingival tissue collected from a healthy patient at the Department of Oral Surgery, Wroclaw Medical University. The experiment was conducted in accordance with the requirements of the Bioethics Committee of Wroclaw Medical University (No. 864/2012). Gingival biopsy, isolation and culture of human fibroblasts from healthy patients were performed as described by Saczko *et al*. (13). Isolated cells were cultivated in DMEM medium (Sigma, Poznan, Poland) supplemented by 5% foetal bovine serum (FBS), (EURx, Gdansk, Poland) and 1% of streptomycin/penicillin (Sigma) in a humidified atmosphere with 5% of CO_2. Before the experiments, cells were washed by Dulbecco's Phosphate Buffered Saline (DPBS), (Sigma) and trypsinized with 0.25% Trypsin-EDTA (Sigma).

Evaluation of collagen content. Sircol™ collagen assay. The Sircol™ collagen assay method (Biocolor Ltd., Carrickfergus, UK) is a color-chemical assay used to determine the content of mammalian collagen type I to V in biological material. It uses the properties of the Sirius Red dye, which binds to the helical structure $[Gly\text{-}X\text{-}Y]_n$ characteristic for all these types of collagen. Primary fibroblasts were prepared and placed into 25 cm^2 culture flasks at 3×10^5 cells/flask in 4 ml of DMEM with 5% serum. After 24 h of incubation at 37˚C, the medium was collected and cells were refed with 4 ml of serum-free DMEM with the addition of the corresponding compound (BE 6 μM, BA 6 μM, Betulin-Dab-NH_2 6 μM). After 3-day incubation at 37˚C and 5% CO_2, the medium was collected and the collagen content was analysed using the Sircol™ Soluble Collagen Assay kit. For analysis 100 μl of media sample were collected, as well as 100 μl of control medium. Collagen solutions (Reference Standard, Biocolor Ltd.) were prepared at the same time to obtain a standard curve in the range of 50-200 μg/ml of collagen. Into test tubes containing medium, control medium, or standard collagen solutions, 500 μl of Sircol Dye Reagent was added (Biocolor Ltd.) and then the tubes were shaken for 30 min. Then, the samples were centrifuged for 30 min at 13000 RCF and 4˚C. The supernatant was then removed and the pellets were resuspended in 500 μl Wash Reagent (Biocolor Ltd.) and centrifuged again at the same conditions. After the supernatant was removed again, the pellet was resuspended in 250 μl Alkali Reagent (Biocolor Ltd.), shaken for 5 min and seeded into a 96 well plate. The absorbance at 550 nm was measured using a multiwell scanning spectrophotometer (EnSpire Multimode Plate Reader, PerkinElmer, Inc. Waltham, MA, USA). Based on the standard curve, the collagen content was calculated in each sample. The effect of the compounds on collagen synthesis was calculated by subtracting from the values of samples those of the control.

Immunocytochemical assay. Expression of type I and III collagen was assessed by immunocytochemistry. For this purpose, human normal fibroblasts were placed onto 8-well microscope slides. After 24 h when the cells were attached to the surface, test compounds were added at a concentration of 6 μM. After a 24-h incubation, the preparations were washed in PBS and then fixed with 4% formaldehyde. The slides were rinsed in PBS buffer and placed in 1% H_2O_2 for 30 min. Then, they were rinsed 3×5 min in PBS with 1% Triton X 100 (Sigma). Next, the slides were drained and a goat polyclonal anti-collagen type I antibody (Santa Cruz BT COL1A1, CA, USA) or a goat polyclonal anti-collagen type III antibody (Santa Cruz BT COL3A1) were applied. After a 24-h incubation at 4˚C, they were rinsed 3x 5 min in PBS with 1% Triton X100. Then the procedure was performed using the DAKO LSAB kit (Carpinteria, CA, USA) + System HRP, successively with Biotinylated Link Universal, Streptavidin –HRP, DAB + substrate buffer with DAB + Chromogen, as described by the manufacturer. For the staining of cell nuclei, hematoxylin (Carl Roth, Karlsruhe, Germany) was used for 30 sec – 1 min. Next, the preparations were rinsed in water for 30 min, carried out by the ascending series of alcohol (50-100%) and dehydrated in xylene. Finally, the preparations were closed with DPX synthetic resin (Aqua Medica, Poland) using cover slides. A

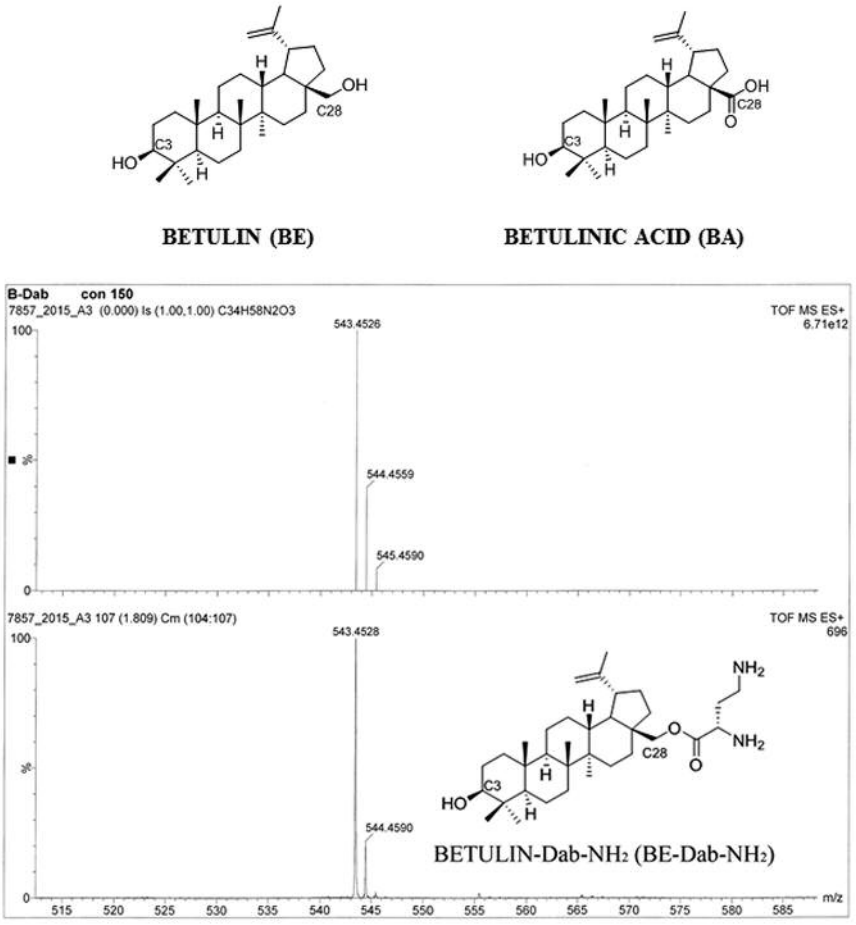

Figure 1. *The chemical structures of betulin (BE), betulinic acid (BA) and betulin ester with L-2,4-diaminobutyl acid (BE-Dab-NH_2). Mass spectroscopy (MS) analysis: MS calculated m/z+=543.4526; MS measured m/z+=543.4528.*

coloured immunocytochemical reaction was assessed using an Olympus BX51 microscope (Japan). The percentage of cells that underwent a staining reaction and the intensity of the immunocytochemical reaction were estimated. The counting was performed by two independent investigators. The intensity of immunohistochemically stained reaction was evaluated as (–) negative (no reaction), (+) weak, (++) moderate, and (+++) strong.

Proliferation test. MTT assay. The effect of the BE-DAB-NH_2, BE and BA on human normal fibroblasts was assessed by the MTT test. The MTT test is a colorimetric method designed to determine the metabolic activity of living cells. Its action is based on the reduction of the tetrazolium salt by mitochondrial dehydrogenase to the coloured crystals of formazanes. The product of this reaction is insoluble in water and therefore requires the delivery of an organic solvent, *e.g.* DMSO or isopropanol. The intensity of the obtained solution translates proportionally to the cell proliferation and is measured spectrophotometrically in the 492-570 nm wavelength range. The cells were seeded into a 96-well culture plate. After 24 h and when the cells were adherent, the culture fluid was removed and the test compounds were added in the appropriate concentration (1 μM, 3 μM, 6 μM, 12 μM, 20 μM). The cells were then incubated 24 h with the test compounds. After this period, the MTT test was performed according to the manufacturer's protocol (Sigma Aldrich, Darmstadt, Germany). After the dissolution of the formazan crystals, the absorbance at 570 nm wave was measured using the Multimode EnSpire Reader (PerkinElmer, Inc.). The absorbance results were

Table I. *Collagen content in the culture medium determined by the Sircol™ Collagen Assay after 72 h of incubation of fibroblasts with the test compounds at a concentration of 6 μM.*

Type of sample	Collagen content [μg/ml]
1. Cells cultured in the presence of BE-Dab-NH_2	64.4±1.2*
2. Cells cultured in the presence of BA	8.2±0.8
3. Cells cultured in the presence of BE	10.2±1.1
4. Control cells	9.4±0.95

*$p<0.001$ with reference to samples 2, 3 and 4 (Student's *t*-test).

Table II. *Evaluation of the intensity of immunocytochemical reaction in human fibroblasts using antibodies against collagen type I and III after 24 h exposure to test compounds at a concentration of 6 μM.*

Type of sample	Type I collagen	Type III collagen
Cells cultured in the presence of BE-Dab-NH_2	98±1.5%*	30%/70±4.3%
	++	++/+++
Cells cultured in the presence of BA	97±2%*	98±1.1%*
	+	+
Cells cultured in the presence of BE	98±1.2%*	99±1%*
	+	+
Control cells	70%/30±2.6%	60%/40±5.1%
	–/+	–/+

*$p<0.05$ with reference to control cells (Student's *t*-test).

converted to a percentage of control cells. The test was performed in triplicate and the percentage of viable cells was determined for individual concentrations of Betulin-DAB-NH_2, BA and BE.

Statistical analysis. Statistical analysis was performed using Statistica 13.0 (Dell Inc., USA). Student's *t*-test, Mann-Whitney *U*-test and nonlinear estimation were used for the calculations.

Results

Mass spectroscopy. The BE-Dab-NH_2 mass spectroscopy analysis is shown in Figure 1. Calculated mass m/z +=543.4526; measured mass m/z +=543.4528.

Sircol™ collagen assay. The collagen content in the medium of fibroblasts incubated with the test compounds was measured by this method. The experiment revealed that fibroblasts cultured in the presence of BE-Dab-NH_2 produced 6.85 times more (increase by 685%) of collagen than control cells, 7.85 times more (an increase of 785%) than cells cultured in the presence of BA and 6.31 times more (increase of 631%) than cells cultured in the presence of BE. At the same time, there was no increase in the collagen content in the BA-treated fibroblast cultures compared to the control, while BE caused a minimal increase in the collagen content compared to the control. Differences in the amount of collagen produced following incubation with BE-Dab-NH_2 in relation to the control, to cells treated with BE and cells treated with BA are statistically significant ($p<0.001$). The results obtained are shown in Table I.

Immunocytochemical staining (ICC). A particularly intense immunocytochemical reaction for both type I and III collagen was found in fibroblasts cultured in the presence of BE-Dab-NH_2. In fibroblasts cultured in the presence of BE or BA, the reaction for both types of collagen was poor, while for control cells, either was lacking or was poor for both types of collagen. The results of the evaluation of the immunocytochemical reaction are presented in Table II. ICC images for control cells and cells incubated with the derivative BE-Dab-NH_2 are presented in Figures 2 and 3.

Proliferation test. The MTT method showed no toxicity of BE, BA and BE-Dab-NH_2 at a concentration of 6 μM, which was used in the experiment with human normal fibroblasts. On the basis of non-linear estimation, the IC_{50} for BE-Dab-NH_2 was calculated which was 31.15 μM. The Mann-Whitney *U*-test showed statistically significant differences in fibroblast proliferation between the concentrations of BE-Dab-NH_2 used: 1 μM and 6 μM ($p<0.05$), 1 μM and 12 μM ($p<0.05$), and 1 μM and 20 μM ($p<0.05$). There was no statistically significant difference in the proliferation of the cells between the individual compounds at a concentration of 6 μM. The results are shown in Figure 4.

Discussion

There are reports regarding stimulation of collagen synthesis by betulinic acid at a concentration of 6 μM (5). The patent states that betulinic acid (BA) has a stronger effect on collagen synthesis than the active ingredients of Centella Asiatica (5). In our experiment, no increase in collagen synthesis was observed after treating fibroblasts with 6 μM BA. Another study on the stimulation of collagen synthesis in human fibroblasts by triterpenes has reported that gedunin (from *Carapa guianensis* seeds) increased collagen synthesis by 130% (14). Triterpene saponins from flowers of *Bellis perennis* also stimulate collagen synthesis in fibroblasts at the level of 130% compared to control (15). *Labisia pumila* is another plant containing triterpene saponins with a documented influence on the synthesis of collagen in human fibroblasts (16).

Betula verrucosa, *Centella asiatica*, *Bellis perennis*, *Carapa guianensis*, *Labisia pumila* are plants used for centuries in folk medicine in various parts of the world to heal wounds and ulcers, and to relieve inflammation of the skin. The active ingredients present in this plant material have been well characterised, but studies examining the

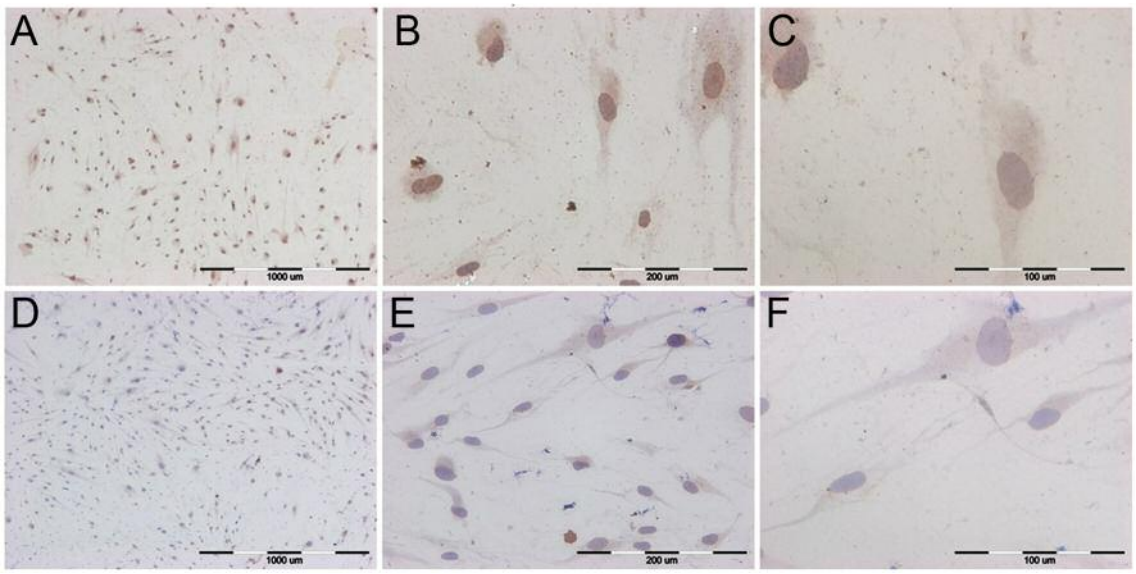

Figure 2. *Photos of fibroblasts with immunocytochemically stained collagen type I. On panels A, B, C are the cells cultured with Betulin-Dab-NH_2 (concentration 6 μM); on panels D, E, F control cells are shown. Magnification on panels A, D ×40; B, E ×200; C, F ×400.*

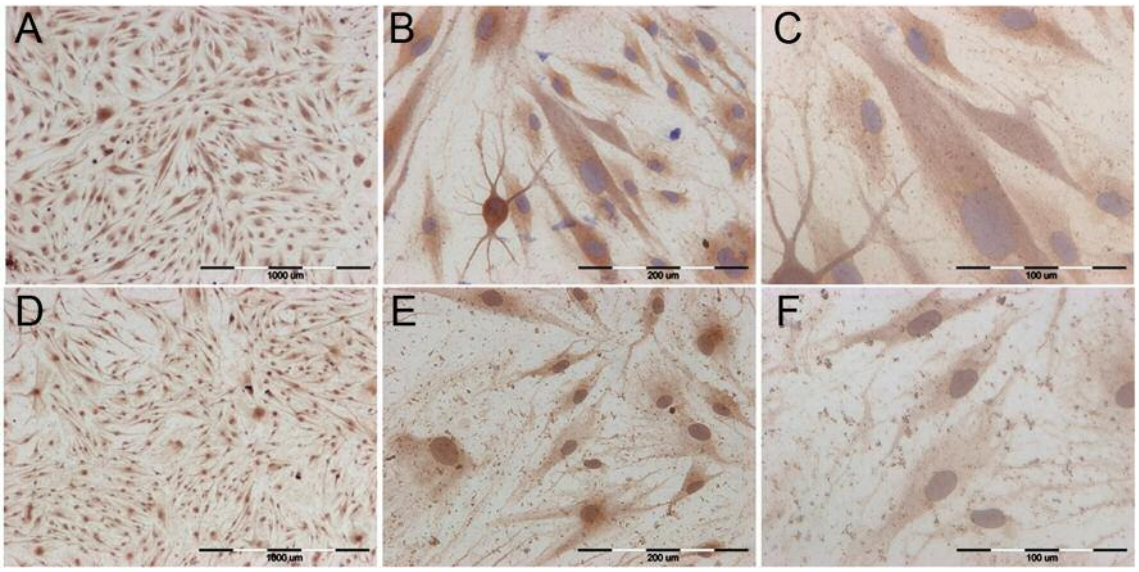

Figure 3. *Microphotographs of fibroblasts with immunocytochemically stained collagen type III. On panels A, B, C are the cells cultured with Betulin-Dab-NH_2 (concentration 6 μM); on panels D, E, F control cells are shown. Magnification on panels A, D – ×40; B, E – ×200; C, F – ×400.*

potential therapeutic properties of individual compounds are still ongoing (14-16). Other studies examining the effect of *Calophyllim inophyllum*, that contains triterpenes, on collagen production by primary fibroblasts have indicated stimulation of proliferation and collagen III production (17).

The European Medicines Agency (EMA) registered in 2016 the drug EPISALVAN (Oleogel-S10, AMRYT) containing 10% of birch bark extract (of which BE is 60-86%) (18). In the second half of 2018, the drug obtained a positive opinion from the FDA (Food and Drug Administration, USA) and is currently in phase III clinical trials in 38 research centres around the world for the treatment of various types of *Epidermolysis bullosa* (EB). EB is a group of genetically determined diseases of the skin

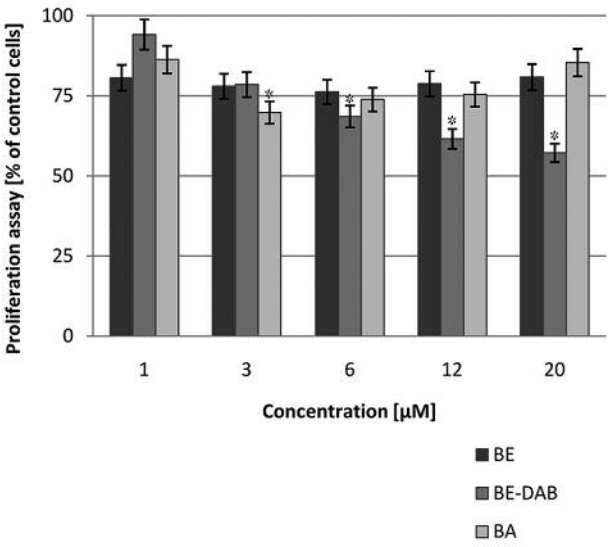

Figure 4. *The effect of BE, BA and BE-Dab-NH_2 on the proliferation of normal human fibroblasts was assessed by MTT. *p<0.05 (Mann-Whitney U-test).*

characterised by the formation of blisters and difficult to heal wounds due to mechanical injuries. Numerous studies have reported on its effectiveness in the treatment of EB wounds and other skin lesions, *e.g.* after laser procedures (19-22). It is the first registered drug containing a formula based on triterpene compounds from birch bark.

In our experiment, the betulin derivative BE-Dab-NH_2 (betulin ester with *L*-2,4-diaminobutyl acid) was shown to have a much greater ability to stimulate collagen synthesis in human fibroblasts than BE and BA. It should also be pointed out that this compound has a much better solubility in water than BE and BA (10). These properties make the new derivative, BE-Dab-NH_2, more appropriate to be included in preparations that heal wounds, regenerate the oral mucosa, and revitalise the skin. A further stage of the current work will be the development of formulations enabling the practical use of our findings in *in vivo* studies and in clinical trials.

Conflicts of Interest

The Authors state that they have no conflicts of interest regarding this study.

Authors' Contributions

MD-Z and JK designed the study. MD and MP carried out botulin derivative synthesis. JK, JS, SB and NR performed the *in vitro* experiments. JK, JS and MD-Z analysed the results. All Authors read and approved the final manuscript.

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