Effect of *Atropa belladonna* L. on skin wound healing: Biomechanical and histological study in rats and in vitro study in keratinocytes, 3T3 fibroblasts, and human umbilical vein endothelial cells

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ABSTRACT

The effect of *Atropa belladonna* L. (AB) aqueous extract on skin wound healing was studied in male Sprague–Dawley rats subjected to two parallel full-thickness skin incisions on the back. Specimens for histological evaluation were collected on days 2 and 5 whereas for biomechanical testing, they were collected on day 5. In the in vitro study, a different concentration of AB extract was used to test the differentiation of keratinocytes using a panel of selected antibodies, proliferation, and cell survival of 3T3 fibroblasts and human umbilical vein endothelial cells using the MTT-assay. Results of the in vivo experiments showed in AB-treated wounds a shortened process of inflammation and accelerated collagen formation, as well as significantly increased wound stiffness as compared with control tissues. The in vitro examination showed that control keratinocytes were cytokeratin 19 free, while samples exposed to the highest AB extract concentration expressed CK19. Moreover, all concentrations were stimulatory to human umbilical vein endothelial cell proliferation. In addition, only the AB extract at the lowest tested concentration increased fibroblast growth, but higher concentrations decreased cell survival. In conclusion, our results indicate that the AB water extract positively affects early phases of skin wound healing in rats. However, the in vitro results on the inverse relation between the concentration of the AB extract and its effects on cell proliferation may be important for future research.

It is well known that delayed wound healing costs the health services a substantial amount of money per year. Therefore, a number of experimental studies deal with new approaches to improve wound healing using either modern physical[1,2] and pharmacological methods[3,4] or phytotherapy.[5,6] Nevertheless, the use of natural products still represents the ultimate option of treatment in many regions of the world. Various extracts from numerous plant families, including Solanaceae, have been described in this context.[7] For instance, *Capsicum annuum* L. and *Solanum incanum* L. extracts have been used in folk medicine to improve wound healing after skin damage.[7]

Atropa belladonna L. (AB)—Deadly nightshade, a member of the Solanaceae family, is a perennial plant widely distributed over Central and Southern Europe. Its basic medicinal properties are attributed to hyoscyamine (and its racemic form atropine) and scopolamin, alkaloids that act as anticholinergic agents by competitively blocking the binding of acetylcholine in the central nervous system and parasympathetic postganglionic muscarinic receptors.[8,9] Historically, physicians have used the preparation of AB and related alkaloids, such as hyosciamine and scopolamine, as soporifics.

Skin wound healing is a dynamic process in which different cell types, such as fibroblasts, leukocytes, and monocytes/tissue macrophages (TM), as well as endothelial cells and epidermal cells cooperate to restore the integrity of an injured body surface. The presence of muscarinic receptors has previously been confirmed in fibroblasts,[10] endothelial cells,[11] and keratinocytes,[12] and thus in cells

involved in the process of skin wound healing. Moreover, some studies have indicated that muscarinic receptors have both inhibitory and stimulatory effects in mouse NIH3T3 fibroblasts[13] and modulate wound reepithelization.[14] From this point of view, it can be hypothesized that AB water extract may also influence skin wound healing.

The use of AB aqueous extract has a long tradition in the Slovak folk medicine. However, the effect of this herb to improve skin wound healing has never been experimentally verified. Therefore, the present investigation was designed to study the effect of AB on the histological and biomechanical (wound tensile strength measurement) parameters of skin wound healing in vivo as well as on the proliferation of 3T3 fibroblasts, human umbilical vein endothelial cells (HUVECs), and on the differentiation of human keratinocytes in vitro.

METHODS

Plant material

AB was collected in August 2006 from the vicinity of mast on "Čertova sihot," Slovak Paradise, Slovak Republic. The plant was identified by Assoc. Prof. Pavol Mártonfi, PhD from the Department of Botany, Institute of Biology and Ecology, P. J. Šafárik University in Košice. A herb of the plant was dried at room temperature in the dark. A voucher specimen (KO-30301) was deposited in the Herbarium of the Botanical Garden of P. J. Šafárik University in Košice.

Preparation of the aqueous extract of AB

Since the AB extract is prepared as a tea in the Slovak folk medicine, some of its components can be destroyed during the preparation. Therefore, we have extracted AB with water as well as ethanol (ETOH) and compared these two methods in an attempt to determine whether plant compounds are altered in a traditional aqueous extract.

A water extract was prepared by pouring 1 g of dried AB leaves with 100 mL (10 mL for in vitro tests) of boiling distilled water. The extract was then left to infuse for 10 minutes at room temperature. The alcohol extract was prepared in mortar by extracting 1 g of dried plant in 10 mL of 96% ETOH. Consecutively, the extracts were filtered (0.2 μm).

High-pressure liquid chromatography (HPLC)

Filtered extracts were analyzed by gradient HPLC, using an SGX C18 7 μm (4×250 mm) column (Tessek, Prague, Czech Republic) at a flow rate of 1.0 mL/minutes. The mobile phase was $A = H_2O$: acetonitrile : H_3PO_4 (80 : 19 : 1) and B=95% acetonitrile. Detection was performed at 210 nm,[15] using detector Ecom LCD 2084 (Prague, Czech Republic). Analyses were replicated three times. Concentration was expressed as mean ± SD (mg/g dry wt.). Atropine and scopolamine (both from Sigma-Aldrich, Prague, Czech Republic) were used as standards.

Animal model

The experimental conditions were in compliance with the requirements of the European rules of ethical standards of animal treatment and welfare. Hence, our experiment was approved by the Ethics Committee of the Faculty of Medicine of Pavol Jozef Šafárik University in Košice and by the State Veterinary Administration of the Slovak Republic.

Male Sprague–Dawley rats (n=80; 8–10 months of age; obtained from the Animal Facility of P. J. Šafárik University) were used for experiments and allocated into eight groups (Table 1). For general anesthesia, a combination of 33 mg/kg ketamine (Calypsol, Richter Gedeon, Budapest, Hungary), xylazine 11 mg/kg (Rometar a.u.v., Spofa, Prague, Czech Republic), and tramadol (Tramadol-K, Krka d.d., Novo Mesto, Slovenia) 5 mg/kg was intramuscularly administered to the rats. Two 4-cm-long parallel full-thickness skin incisions were performed under aseptic conditions on the left and right side of each rat spine and immediately closed using an intradermal running suture (Chiraflon 5/0, Chirmax, Prague, Czech Republic).

Wound treatment

During the treatment, all rats were restrained individually in a plexiglass cage with a circular opening over the wounds. However, in the control groups, the AB aqueous extract was not applied. In the experimental groups, a 1% extract was applied three times (at 8-hour intervals) a day at group-dependent time intervals (Table 1). Application of the extract with a gauze sponge lasted 10 minutes. To prevent the possible thermic effect of the application, the extract temperature was approximately 37 °C.

Table 1. Dividing of animals into 10 groups (H, histological evaluation; B, biomechanical evaluation; C, control group; T, treated group); application scheme of 1% *Atropa belladonna* L. water extract during the experiment

	No. of treatments with *A. belladona* water extract per day					
Group	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5
H2-C (n=10)	—	—	Killed	—	—	—
H2-T2 (n=10)	3	3	Killed	—	—	—
H5-C/B5-C (n=10/n=10)	—	—	—	—	—	Killed
H5-T2/B5-T2 (n=10/n=10)	3	3	—	—	—	Killed
H5-T5/B5-T5 (n=10/n=10)	3	3	3	3	3	Killed

Wound tensile strength measurement

This method was described in our previous study.[16] Briefly, a device for measuring wound-breaking strength included a stand with a moving arm that transfers force from the sample to a piezoelectric sensor FSG15N1A (Honeywell, Morristown, NJ). The sensor–computer interface module ADAM 4011 was used (Advantech, Irvine, CA). To achieve vertical tensile force, a servomechanism (power supply: ±3 V; output force: 0–30 N) was used.

The suture was removed and using a template, each skin area with a wound was adjusted to a 3×2 cm (length of measured wound=2 cm) strip to obtain uniform samples. The samples were placed between the two clamps of the tensiometer. The maximal breaking strength (MBS) was registered for each sample.

The tensile strength of wounds was calculated using the formula: TS=MBS/A (TS is tensile strength in [g/mm^2], MBS is the maximal breaking strength [g], A is the wound area [mm^2]). Each measurement was performed blind.

Basic histology and immunohistochemistry

Tissue specimens were processed routinely for light microscopy (fixation in 4% buffered formaldehyde, dehydration, paraffin embedding, sectioning [5 μm], and staining). Hematoxylin–eosin was used for basic staining, and the van Gieson method was used for nonspecific collagen staining.

Cytokeratin (CK)10, which is the main suprabasal marker of differentiation[17,18] and is expressed during normal keratinocyte differentiation, was detected using mouse monoclonal antibody (DAKO, Brno, Czech Republic). Swine-anti-mouse immunoglobulin labeled by fluorescein isothiocyanate (FITC; AlSeVa, Prague, Czech Republic) was used as the secondary antibody. Control of the specificity was performed by replacement of the first step antibody by a monoclonal antibody of the same isotype directed against the antigen not occurring in the epidermis. The nuclei of the cells in histological sections were counterstained with 4′,6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich), specifically recognizing DNA.

Semi-quantitative analysis of histological sections

A semi-quantitative method[19,20] was used to evaluate reepithelization and keratinization of the epidermis; the presence of inflammatory cells (polymorphonuclear leukocytes [PMNL]); fibroblasts; creation of a new extracellular matrix (ECM)—especially new collagen; and the presence of new vessels. Sections were evaluated in coded slides according to the scale 0, 1, 2, 3 (Table 2).

Isolation and in vitro cultivation of keratinocytes

Keratinocytes were isolated from the residual skin samples, which were obtained from the Department of Aesthetic Surgery of the 3rd Faculty of Medicine of Charles University according to the criteria of Helsinki Declaration with informed consent of patients approved by the local Ethical Committee. The skin graft was treated overnight with 0.3% solution of trypsin at 4 °C. Keratinocytes obtained from the epidermis were expanded following the modified Rheinwald–Green method.[21] Briefly, prior cocultivation with keratinocyte proliferation activity of fibroblasts was stopped using a solution of Mitomycin C (Sigma-Aldrich) at a concentration of 25 μg/mL for 3 hours. Feeder cells were seeded on a cover glass at a density of 25,000 cells/cm^2 and cultured for 24 hours. A suspension of keratinocytes (20,000 cells/cm^2) was then added and cells were cultivated in a keratinocyte medium at 37 °C and 3.3% CO_2. The culture medium contained tested chemicals at final concentrations of 1, 0.33, and 0.11%.

Table 2. Scale for the semi-quantitative assessment of histological sections

Scale	Epithelization	PMNL/fibroblasts/collagen/vessels
0	Thickness of cut edges	Absent
1	Migration of keratinocytes	Mild
2	Bridging of the incision	Moderate
3	Keratinization	Marked

PMNL, polymorphonuclear leukocytes.

Immunocytochemistry of in vitro cultured keratinocytes

The cells adhering to the coverslips were washed in phosphate-buffered saline (PBS) and fixed briefly with 5% paraformaldehyde diluted in PBS (pH=7.3). CK19, which is known to be expressed in poorly differentiated keratinocytes as stem cells,[22] and pankeratin were detected in cultured keratinocytes simultaneously.[23] Monoclonal antibody against keratin 19 was purchased from DAKO and the monoclonal antibody against pankeratin from AbCam (Cambridge, UK). FITC-labeled swine-anti-mouse (SwAM-FITC, AlSeVa) and tetramethyl rhodamine iso-thiocyanate (TRITC)-labeled goat-anti-mouse (Sigma-Aldrich) sera were used as a second step antibody. Both the primary and the secondary antibodies were diluted as recommended by the supplier. The reaction specificity was tested by replacement of a distinct antibody by another polyclonal or monoclonal antibody of the same isotype, but against antigens not present in the studied cells. The nuclei of the majority of specimens were counterstained with DAPI (Sigma-Aldrich), specifically recognizing DNA. The specimens were mounted to Vectashield (Vector Laboratories, Burlingame, CA).

Both skin sections and cultured cells were analyzed by fluorescence microscopy using a Nikon Eclipse 90i apparatus (Nikon, Prague, Czech Republic) equipped with filterblocks specific for FITC, TRITC, and DAPI, respectively, a high-resolution CCD camera Cool-1300Q (Vosskühler, Osnabrick, Germany), and LUCIA 5.1 software (Laboratory Imaging, Prague, Czech Republic).

In vitro cultivation of 3T3 fibroblasts

A standard laboratory cell line of 3T3 fibroblasts was used for the experiment. Fibroblasts were cultured in

Dulbecco's modified Eagle's medium (DMEM) (Gibco Laboratories, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) and antibiotics (streptomycin and penicillin, both from Gibco Laboratories).

Isolation and in vitro cultivation of HUVECs

HUVECs were isolated from freshly collected human umbilical cords using collagenase type II (Gibco Laboratories) digestion of the umbilical vein according to the previously described method by Jaffe et al.[24] Briefly, first, the umbilical cords were thoroughly flushed with buffer (0.14 M NaCl, 0.00052 M Na_2HPO_4, 0.00015 M KH_2PO_4, and 0.011 M glucose in distilled water), then the vein was filled with 0.1% collagenase type II (Gibco Laboratories) solution, clamped, and incubated at 37 °C for 15 minutes. After incubation, one end of the umbilical cord was cut and the vein was rinsed by perfusion with culture medium. The effluent with cells was collected and centrifuged. Cells were plated in 100×20 mm tissue culture dishes (Sarstedt, Nümbrecht, Germany) coated with 1.5% gelatin. Cells were grown to confluence in the Medium 199 supplemented with 20% heat-inactivated FBS, streptomycin, and penicillin (all from Gibco Laboratories). The endothelial identity of the cells was confirmed by their "cobblestone" morphology and CD31 expression as determined by flow cytometry. The cells were stained with a CD45-FITC (BD Biosciences, Rockville, MD)/CD31-PE (Caltag Laboratories, Burlingame, CA) combination of monoclonal antibodies and analyzed using a FACS Vantage SE flow cytometer (BD Biosciences). Primary cultures were harvested at confluence with 0.05% trypsin-0.02% ethylenediaminetetraacetic acid (EDTA; Gibco Laboratories) and plated at a split ratio of 1 : 3 in tissue culture dishes. Subconfluent cells were allowed to grow to confluence under the same conditions, harvested during the exponential cell-growth phase with trypsin-EDTA.

Metabolic assay of 3T3 fibroblasts and HUVECs

For the assessment of cell survival and proliferation, the thiazolyl blue (MTT) method was used.[25] Briefly, per well, 2×10^4 HUVECs and 1×10^4 3T3 cells were plated in 96-well polystyrene microplates (Sarstedt), respectively. The culture medium contained the tested chemicals at final concentrations of 1, 0.5, 0.25, 0.125, and 0.0625%. After 3 days of incubation, 10 μL of MTT (5 mg/mL) (Sigma-Aldrich) was added to each well. After an additional 4 hours, during which insoluble formazan was produced, 100 μL of 10% sodium dodecylsulfate was added to each well. During the next 12 hours, the formazan was allowed to be dissolved. The absorbance was measured at 540 nm using an automated MRX microplate reader (Dynatech Laboratories, Billingshurst, UK). The absorbance of the control wells was taken as 100% and the results were expressed as a percentage of the control. Each experiment was repeated three times.

Migration/wound-healing assay of 3T3 fibroblasts and HUVECs

Subconfluent monolayers in 24-well plates were wounded with pipette tips, giving an acellular 1-mm-wide lane per well. After washing, cells were supplied with 1.5 mL cultivation medium in the absence (controls) or in the presence of AB. The culture medium contained the tested chemicals at final concentrations of 1, 0.33, and 0.11%. HUVECs and 3T3 fibroblasts were then allowed to migrate into the wound over a 12- and 24-hour period, respectively. Quantification of cell migration was performed by measuring the diameters of wounds using the QuickPHOTO MICRO 2.2 (Promicra, Czech Republic) software. Each experiment was repeated three times.

Antimicrobial tests

The antimicrobial activity of the AB water extract was tested against the following strains—*Bacillus subtilis* (CCM 4062), *Enterococcus faecalis* (CCM 4224), *Escherichia coli C 600 Rif* (CCM Ec 336/77), *Micrococcus lysodeicticus* (CCM 410), *Pseudomonas aeruginosa* (CCM 3955), *Staphylococcus aureus* (CCM 4223), and *Streptococcus pyogenes* (CCM 4425)—which were obtained from the Czech Collection of Microorganisms (CCM, Czech Republic). Minimal inhibitory concentrations (MIC) were estimated by the microdilution assays M27-A and M38-P NCCLS (National Committee for Clinical Laboratory Standards, USA). Antibiotic ciprofloxacin (Ciprinol, Krka d.d.) was used as a positive control.

Statistical analysis

For each parameter, average values with standard deviations (mean ± SD) were calculated. One-way analysis of variance, followed by Tukey–Kramer multiple comparison tests, were used to compare the differences in wound tensile strengths and MTT tests. To compare the data obtained from the semi-quantitative analysis, Mann–Whitney and Kruskal–Wallis tests were used. For each test, significance was accepted at $p < 0.05$.

RESULTS

HPLC

Comparison of the aqueous and alcoholic plant extracts using HPLC showed peaks with the same retention times in both extracts and pointed out that the extract included at least three main components. Since alcohol was previously considered to be too aggressive for wound treatment,[26] we chose the water extract to be tested as a promoter of skin wound healing.

Figure 1 shows a chromatogram of water extracts prepared from leaves of AB. The concentrations of selected tropane alkaloids in the water extract from leaves were 0.510 ± 0.086 mg/g dry wt. for atropine, and 0.0406 ± 0.0053 mg/g dry wt. for scopolamine.

Wound tensile strength

Figure 2A shows the results of wound TS measurements on day 5. The lowest wound TS were observed in the B5-C group (8.5 ± 1.6 g/mm^2). Higher wound TS results were found in rats treated for 2 days in B5-T2 group (10.3 ± 2.0 g/mm^2), while the highest wound stiffness was measured in animals from the B5-T5 group treated for 5

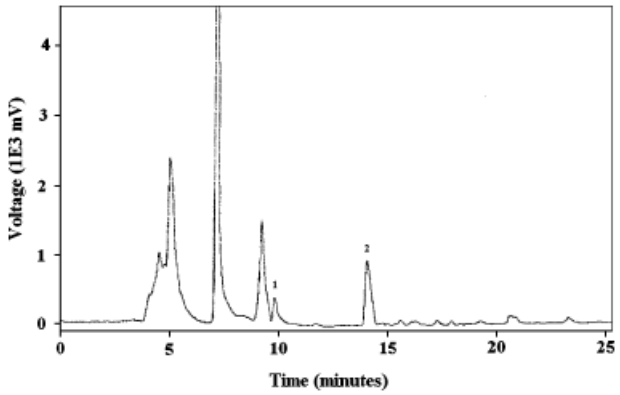

Figure 1. High-pressure liquid chromatogram of water extract of *Atropa belladonna* L. leaves; detection at 210 nm (1—scopolamine, 2—atropine).

days ($10.7 \pm 2.5\,g/mm^2$). The differences between the control group and both experimental groups were statistically significant (B5-C vs. B5-T2 $p < 0.05$; B5-C vs. B5-T5 $p < 0.01$).

Histology

The results from the semi-quantitative analysis of histological sections are summarized in Table 3.

The histological sections from the experimental animals killed 2 days post wounding showed a significantly lower number of inflammatory cells than controls. As compared with control wounds, progress in the healing of AB-treated wounds was seen in the epidermis. The control incisions were not always bridged by a layer of epithelial cells (Figure 3A), while the treated wounds were in most cases completely bridged by two to three layers of epithelial cells (Figure 3C). If wounds, whether control or treated, were bridged by a new epithelial layer, CK10 was expressed (Figure 3B and D), which showed a normal level of keratinocytes differentiation in all rats. In addition to epidermis regeneration, significantly increased angiogenesis was observed in treated rats when compared with their non-treated controls.

Histological analysis of sections obtained 5 days after wounding showed that the acute inflammatory process in all groups was in its final phase. PMNL were only randomly dispersed in the dermis and there was a moderate predominance of tissue macrophages. Keratinocytes migrated beneath the scab, and completely bridged the whole incisions. In most control and in all experimental samples, the differentiation process of keratinocytes was confirmed by the appearance of a keratin layer (cells without nuclei) above the epithelial layers (cells with nuclei). However, no significant differences were found in any of the groups in this time period. The sections showed a typical histological picture of the proliferative phase of healing, with expressive representation of fibroblasts and new vessels (in the deepest parts of the wounds). In addition, the collagen fibers in the control group were only randomly organized and occupied the space between cells in the newly formed ECM. Moreover, the amount of collagen in the incisional space significantly increased in both treated groups when compared with their controls (Figure 3E–G; Table 3).

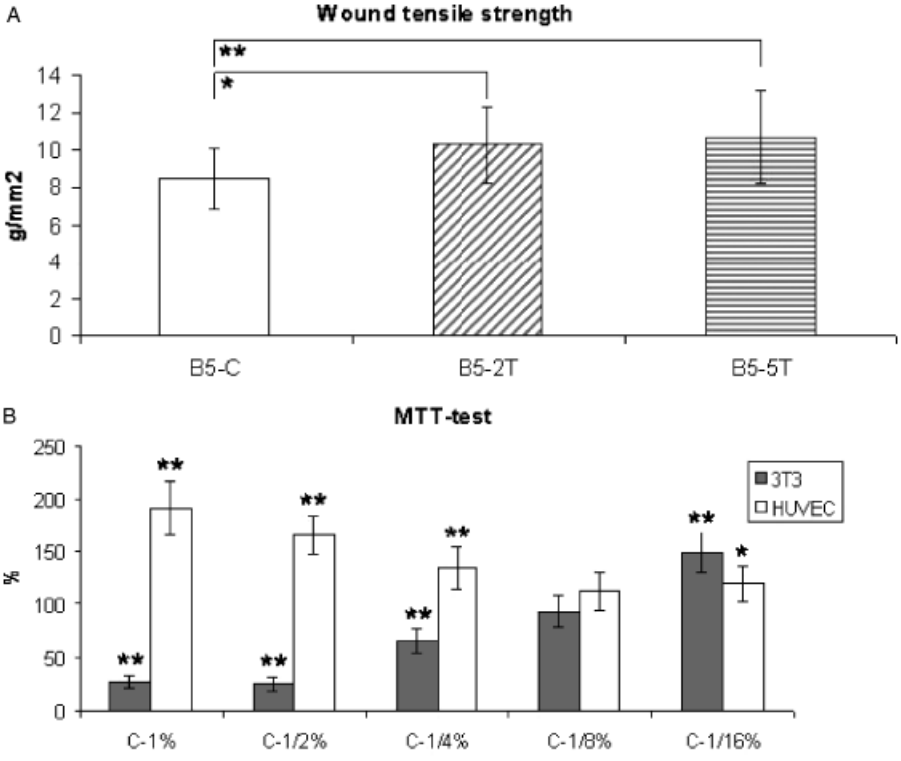

Figure 2. The upper graph (A) shows the wound tensile strength of animals killed 5 days after surgery, i.e., controls (B5-C), 2 days treated (B5-T2), and 5 days treated (B5-T5). The lower graph (B) shows the proliferation of 3T3 cells and human umbilical vein endothelial cells (HUVECs). The controls are taken as 100% and the results of different tested concentrations of *A. belladonna* were expressed as a percentage of the control; (*$p < 0.05$; **$p < 0.01$).

Table 3. Results (median values) from the semi-quantitative evaluation of selected histological parameters/changes in control and treated wounds

Parameter/group	H2-C	H2-T2	H5-C	H5-T2	H5-T5
Reepithelialization	1.5	2*	3	3	3
PMNL	2	1.5**	0	0	0
New collagen	0	0	2	3*	3*
Fibroblasts	1	1*	3	3	3
New vessels	1	1*	2.5	3	3*

*$p < 0.05$; **$p < 0.01$.
PMNL, polymorphonuclear leukocytes.

In vitro cultured keratinocytes

The immunocytochemical staining of the CK19 showed clear differences. Whereas the control cells were CK19 free (Figure 4D), samples exposed to the highest concentration of AB expressed CK19 (Figure 4F). Nevertheless, the proliferation of keratinocytes decreased with increasing concentration of plant's extract (Figure 4A and C).

3T3 fibroblasts and HUVECs

Survival of 3T3 fibroblasts and HUVECs exposed to various concentrations of the tested agent is shown in Figure 1B. The aqueous extract of AB significantly decreased 3T3 cell survival at higher concentrations. However, at the lowest concentration, AB increased cell growth. At the highest concentration, AB was the most potent accelerator of HUVEC growth. The extract increased cell proliferation up to 200% of the control value and the extract's activity decreased parallel with its concentration.

The migration of HUVECs was accelerated using all concentrations of AB (Figure 5). In contrast, the 3T3 cells were accelerated using the lowest concentration while higher concentrations decreased the migration activity (Figure 5).

Antimicrobial properties of AB aqueous extract

The results of the antimicrobial tests are summarized in Table 4. The tests showed that the AB water extract has no antimicrobial properties against bacteria that may occur in wounds. Nevertheless, the extract inhibited the proliferation of *Micrococcus lysodeicticus* and *Bacillus subtilis*.

DISCUSSION

It is well known that during epidermis regeneration and also during many pathological conditions (carcinoma development, etc.), keratinocytes become activated. During these processes cells are characterized by the production of specific keratin proteins that reflect their level of differentiation.[27] Among these proteins, CK1 and CK10 have been considered as early markers of keratinocyte differentiation.[28] Therefore, early detection of CK10 in our study in both control and treated wounds indicates that the process of epidermal cell differentiation is not inhibited; thus,

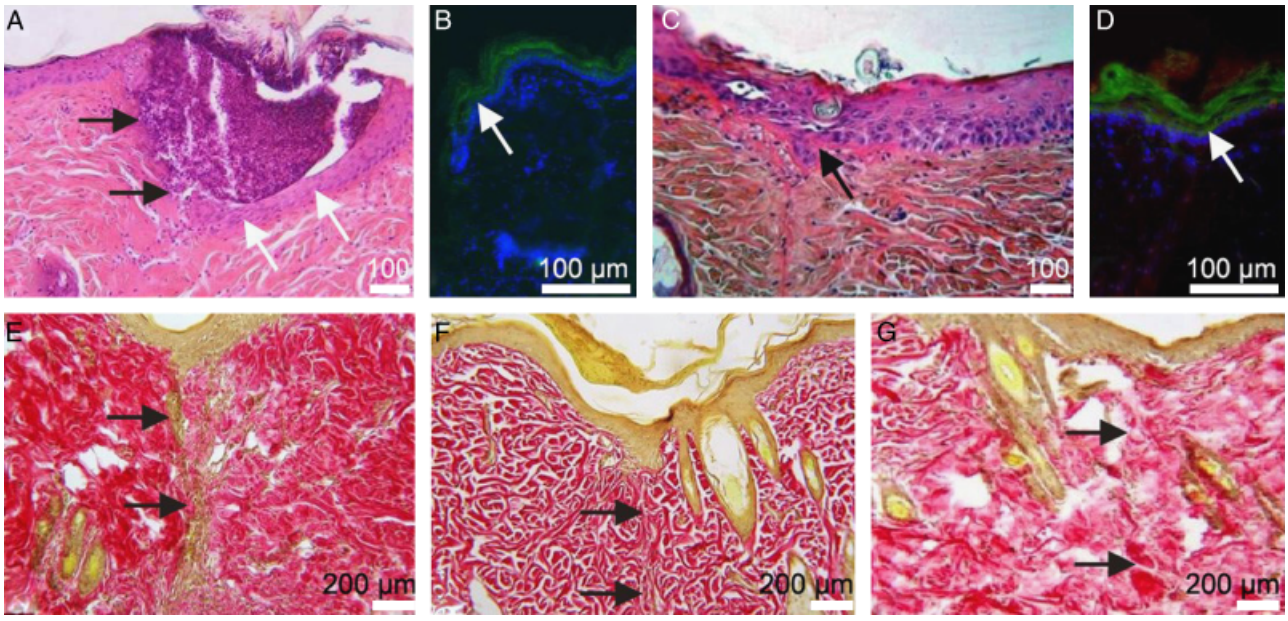

Figure 3. Wounds at 48 hours after surgery (×200; hematoxylin & eosin staining). (A) Control wound, formation of the finished demarcation line (black arrows), migration of epithelial cells beneath the scab (white arrows). (B) Migrating cells express cytokeratin 10 (arrow). (C) Treated wound, wound bridged by 2–3 layers of epithelial cells (arrow). (D) Cells express CK10 over the entire incision (arrow). Wounds at 120 hours after surgery (×100; VG staining). (E) Control wound, incisional gap without significant quantity of collagen (arrows), (F) 2 days treated wound, new formed extracellular matrix in the incisional space containing newly created collagen (arrows). (G) Five days treated wound, new collagen in the incisional space (arrows).

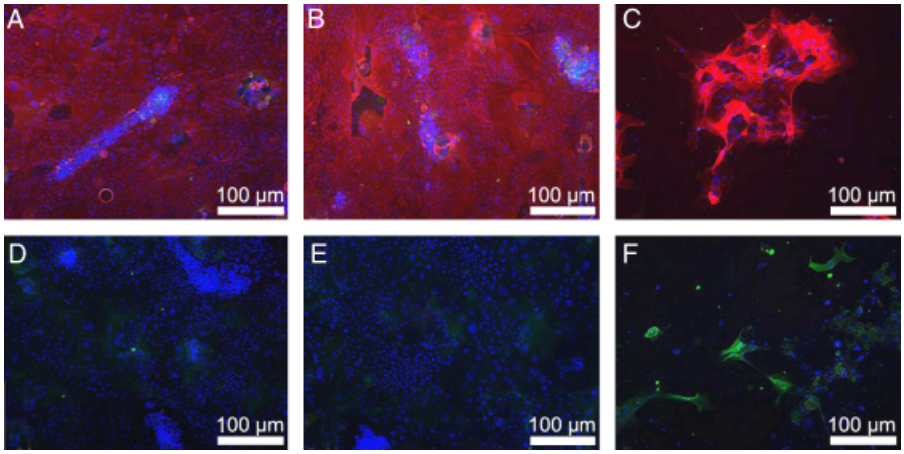

Figure 4. (A,B,C: cells stained for keratins; D,E,F cells stained for CK19). (A and D) Control keratinocytes. (B and E) Keratinocytes cultured with the lowest tested concentration of *Atropa belladonna* water extract. (C and F) Keratinocytes cultured with the highest tested concentration of plant's extract. (A, B, and C) The figure shows that increasing concentration of the tested extract decreased the proliferation of keratinocytes. (F) Only cells cultured with the highest tested concentration express CK19. Nuclei of all cells stained with 4′,6-diamidino-2-phenylindole.

the tested extract does not impair epidermis regeneration in vivo.

Previously, it was reported that M_3 and M_4 muscarinic receptor-subtypes had different influences on wound reepithelization.[14] In M_3 knockout mice, an accelerated process of keratinocyte migration was observed while in M_4 knockout mice the epithelization was inhibited. From this point of view, compounds included in the AB water extract, such as atropine, hyoscyamine, and/or scopolamine, may modulate different muscarinic receptor subtypes with different affinities. This may be the reason why the effect of plant's extract on epidermis regeneration in the present experiment is significant and induced the expression of epidermal stem cells marker CK 19 in vitro,[22,29] but decreased the proliferation of keratinocytes in the culture with increasing extract concentration. However, only cells exposed to the highest tested concentration of AB expressed CK19, while the proliferation of these cells decreased. This observation may also be partially explained by the negative effect of AB at such a concentration on fibroblasts that were used as a feeder layer.

It was found that wound treatment with *Datura alba* L. (Solanaceae) led to accelerated epidermis regeneration, increased cellular infiltration (inflammatory cells), and proliferation (fibroblasts and endothelial cells).[30] Moreover, these results indicated that the positive influence may not only be mediated through the angiogenic and mitogenic effects of the plant but also through its significant antibacterial properties. This is partially in agreement with our results in which *A. belladonna* (Solanaceae) significantly reduced the process of inflammation, but had no significant antibacterial effect on the tested bacteria species. Moreover, the anti-inflammatory properties of AB were supported by significantly increased wound tensile strength in both treated groups just with unimpressive treatment duration differences.

Interestingly, our in vitro study indicated that HUVEC and 3T3 cells displayed an inverse relation to the tested

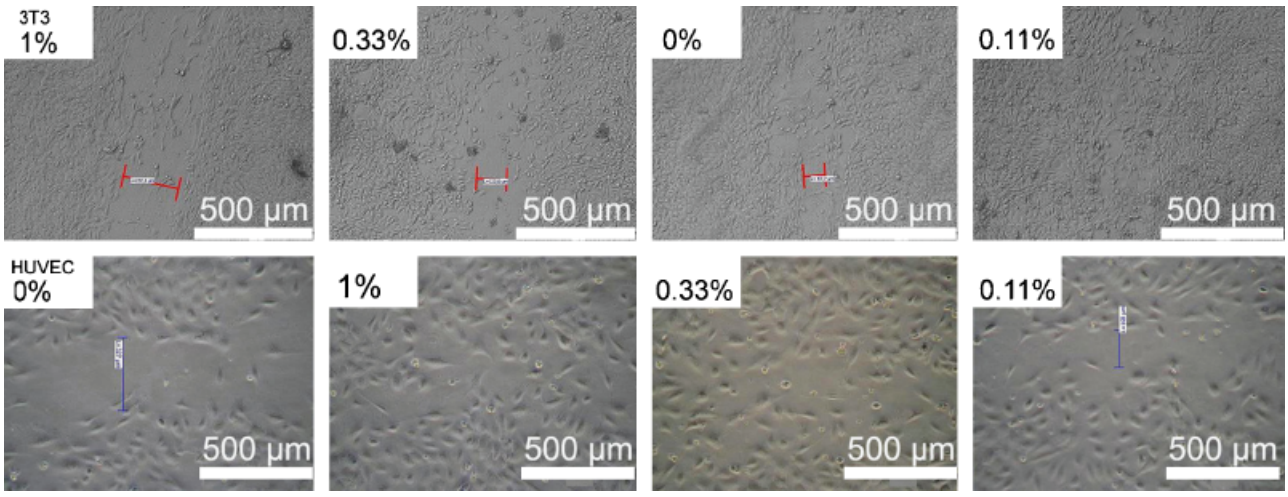

Figure 5. Migration/wound-healing assay of 3T3 cells and human umbilical vein endothelial cells (HUVECs). Migration of HUVECs (lower line of figures) was accelerated by using all concentrations of *A. belladonna* while 3T3 fibroblasts (upper line of figures) were accelerated only by using the lowest concentration (scale 500 µm).

Table 4. Antimicrobial tests of selected bacteria (MIC, minimal inhibitory concentration, n, no antimicrobial properties at any tested concentration)

Tested microorganism	*A. belladonna* MIC (μg/mL)	Ciprofloxacin MIC (μg/mL)
Bacillus subtilis	10×10^3	0.25
Enterococcus faecalis	n	4
E. coli C 600 Rif.	n	≤0.062
Micrococcus lysodeicticus	1.25×10^3	0.5
Pseudomonas aeruginosa	n	≤0.062
Staphylococcus aureus	n	1
Streptococcus pyrogenes	n	0.5

A. belladonna, *Atropa belladonna*; *E. coli*, *Escherichia coli*.

concentrations. From this point of view, the AB concentration for clinical use should be selected meticulously. In contrast to our results concerning the positive effect of AB on angiogenesis, Mathur et al.[51] recorded inhibition of vascular endothelial growth factor-induced capillary formation by a chloroform extract of *Withania somnifera* L. (Solanacea) roots in vivo. In addition, in our animal study, AB significantly increased the number of fibroblast in the dermis at day 2, but had both stimulatory as well as inhibitory activity (concentration dependent) on 3T3 cells in vitro. Therefore, further animal studies focused on additional extract concentration need to be performed. Interestingly, it was found that carbachol (muscarinic receptor agonist) stimulates lung fibroblast proliferation[32]; thus, the question of whether fibroblast modulation by AB (muscarinic receptors antagonist) is linked through muscarinic receptors remains open.

In conclusion, our study shows that an aqueous extract of AB positively modulates early phases of skin wound healing without significant antimicrobial properties. Our findings also indicate that the plant's effect is based on the acceleration of angiogenesis and on its anti-inflammatory properties. Nevertheless, further research is needed to address the precise underlying mechanisms of its action and to find the optimal therapeutic concentration for use in clinical practice.

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