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In Vitro Toxicity Evaluation of Some Plant Extracts and Their Potential Application in *Xerosis cutis*

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Abstract: *Xerosis cutis* represents one of the most common dermatological diagnoses, which, when untreated, can be the trigger for open wounds, infections, and other skin diseases. Plant extracts are a valuable option for long-term treatments for xerosis due to their phytocompounds, especially polyphenols, flavonoids, triterpenes, and polysaccharides, with antioxidant, anti-inflammatory, antimicrobial, moisturizing, and reparatory effects. Active substances have different mechanisms; therefore, evaluating the effects on the cells can be a key indicator, providing valuable information in terms of both cytotoxicity and efficacy. The in vitro and in vivo toxicity tests performed for *Betulae extractum*, *Liquiritiae extractum*, and *Avenae extractum* highlighted potential toxic effects at higher concentrations in a dose-dependent relationship, but at lower levels they can be considered safe (12.5 µg/mL for birch and licorice extracts, 50 µg/mL for oat extract). Concerning the re-epithelialization process, the results revealed that all three dry extracts effectively stimulate skin cell migration, highlighting a potential anti-inflammatory effect by increasing the cell migration rate in conditions of induced inflammation associated with oxidative stress. Among the tested concentrations with a potential contribution to wound healing, the following standout: are birch bark extract 3 µg/mL, licorice root extract 7.5 µg/mL, and oat herb (harvested before flowering) extract 7.5 µg/mL.

Keywords: *Betulae extractum*; *Liquiritiae extractum*; *Avenae extractum*; cytotoxicity; *Daphnia* sp.; cell migration; keratinocytes

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1. Introduction

The appearance and integrity of the skin are the results of a balance between the level of hydration and the intercellular lipid composition, the skin's health being an indicator of the body's overall health [1]. Disturbances in this balance lead to physiological changes, with the onset of skin pathologies, including xerosis [2]. *Xerosis cutis* (xeroderma, asteatosis) is a skin condition resulting from a hydrolipid deficiency, with a stiffening and embrittlement of the corneocytes envelope [3]. It represents one of the most common dermatological diagnoses, with an essential psychosocial impact and a worldwide prevalence estimated

between 29% and 85%, depending on the age, occupational factors, underlying medical conditions, etc. [4–6].

The main objective of current therapies regarding moisturizing products is to adapt the treatment to the distinct abnormalities manifested by the generally recognized symptoms, classified into four stages of xerosis: mild (with a tendency to dryness), moderate (with mild dryness), severe (with moderate dryness), and very severe (with severe dryness, inflammation) [5,7]. In severe forms, symptoms are exacerbated, with the formation of macro fissures associated with inflammation, itching, and erythema. Untreated, these fissures deepen into the skin, eventually becoming open wounds, increasing the risk of bleeding and infection [8]. Recommended for the treatment of fissures are products with active ingredients that develop moisturizing and repairing effects, in the sense of stimulating the migration of keratinocytes from the damaged epidermis, with the formation of new tissue (re-epithelialization) [5], thus restoring the barrier function and, at the same time, the skin homeostasis [9].

In this regard, plants represent an important source of active principles with therapeutic potential. With an extensive background, bioactive extracts obtained from natural sources are used in formulations for topical application to alleviate skin diseases due to their phytocomplexes rich in a wide variety of compounds, especially polyphenols, flavonoids, phenolic acids, and triterpenes, due to their antioxidant, anti-inflammatory, antimicrobial, antiallergic, immunomodulating, and photoprotective qualities; thus, plant extracts can be considered a valuable option for long-term treatments for xerosis [10–15].

Because not all the active substances have the same mechanism or impact level, evaluating the effect on the cells can be a significant indicator, providing valuable information. In this regard, in the development process of topical products, the safety assessment through cytotoxicity assays represents a critical step for a better knowledge of the active ingredient regarding the therapeutic interval, which is essential for further formulation [16,17]. The toxicity can be developed through several mechanisms, membrane integrity being the most frequently used characteristic [18]. In order to determine the possible negative impact (the capacity to affect cellular growth and to produce cellular damage), two important in vitro tests can be performed—the cell viability and cytotoxicity assays, together being considered an initial indication of whether a substance could be or not toxic in vivo [19,20].

Three plant products were selected for the therapeutic approach of xerosis, based on the existing data in the specialized literature regarding their phytocomplexes and therapeutic effects: *Betulae cortex*, *Liquiritiae radix*, and *Avenae herba* (harvested before flowering). The overall extraction process conditions applied for obtaining the studied dry extracts and the phytochemical screening performed previously [21] demonstrate that the three extracts possess great total phenolic compounds content (polyphenols, flavonoids, and phenol carboxylic acids) and the presence of triterpenes, polysaccharides, amino acids, and fatty acids, compounds with significant impact on damaged skin through their effectiveness as antioxidants, as anti-inflammatory, and antibacterial agents, and due to their potential to stimulate re-epithelialization.

Concerning the ability of extracts to stimulate cell proliferation, antioxidant activity plays a key role. Previously, in silico molecular docking highlighted the great potential of the three extracts to exert antioxidant and anti-inflammatory effects in vivo due to their ability to stimulate the Keap1-Nrf2 pathway [21]. Thus, there is a need for a multifaceted approach that integrates different assays, including cellular models, in addition to chemical techniques [22]. The tests performed for evaluating cellular antioxidant activity are biologically relevant methods due to the advantage of using specific human cell lines with physiological importance, based on the interaction between the tested biocomplex and complex enzymatic reactions in a biological system [23–26]. Furthermore, studies of in vitro wound healing models (scratch test) have highlighted some relevant mechanisms in the wound repair process. Also, being an important tool to study the impact of specific compounds on the events occurring during the re-epithelialization process, it opens a way to improve clinical treatments [8].

In our studies, for the evaluation of the cytotoxicity potential of plant extracts and their effects on cell viability, we applied two in vitro tests, respectively: an MTS assay (reduction in the tetrazolium salt), and LDH assay (the release of the cytosolic enzyme lactate dehydrogenase into the extracellular media). Also, the in vivo acute toxicity was investigated using two *Daphnia* species, *Daphnia magna* and *Daphnia pulex*, and the potential teratogenic effects were explored through an embryo test conducted on *Daphnia magna* embryos. In order to obtain information about the effectiveness of phytocomplexes in damaged skin tissue, the antioxidant potential of the bioactive constituents was evaluated (Total antioxidant capacity assay—TAC assay), as well as the efficacy of the extracts through an in vitro cell migration test ("scratch-test"), both tests being performed on standardized normal human keratinocyte cell lines. The studied extracts' toxicity and cell migration-stimulating tests represent the starting point in developing topical products customized for the xerosis stages based on the characteristic symptoms.

2. Materials and Methods

2.1. Reagents

The CytoTox 96® Non-Radioactive Cytotoxicity Assay kit [27] and CellTiter 96™ AQueous One Solution Cell Proliferation Assay kit [28] were purchased from Promega Corporation, Madison, WI, USA, and the OxiSelect™ Total Antioxidant Capacity (TAC) Assay Kit [29], from Cell Biolabs Inc., San Diego, CA, USA. High-glucose Dulbecco's Modified Eagle's Medium (DMEM) was purchased from HyClone® (Thermo Fisher Scientific, Logan, UT, USA), fetal bovine serum (FBS) and trypsin-Ethylenediaminetetraacetic acid (EDTA) (0.25%), from Gibco® (Thermo Fisher Scientific, Logan, UT, USA), and the antibiotic-antimycotic solution (100×) was purchased from Thermo Fisher Scientific, Logan, UT, USA. The epidermal keratinocytes (HaCaT) cell culture was purchased from ThermoFisher Scientific.

2.2. Cell Culture

For in vitro cytotoxicity assays, HaCaT cells were seeded in 96-well tissue culture plates, at the density of 7.5×10^3 cells per well, in high-glucose Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10% fetal bovine serum and 1% antibiotic-antimycotic solution. Cells were maintained at 37 °C under a humified atmosphere containing 5% CO_2. For passaging, cells were detached with a trypsin-EDTA solution. After 24 h, the test protocols were applied.

Regarding the total antioxidant capacity assay, HaCaT cells were seeded in 12-well tissue culture plates at the density of 10^5 cells per well in the same conditions as above. The test protocol was also applied after 24 h.

For the cell migration assay, cells were seeded in untreated 96-well flat-bottom plates at the density of $15–25 \times 10^3$ cells per well for 24–48 h (until the cell monolayer is 100% confluent). After the scratching stage, the cells were monitored for 24 h in the presence of the tested extracts and specific stimuli.

2.3. Dry Hydroalcoholic Extracts

In order to perform the in vitro tests, the three dry extracts (*Betulae extractum*—BE, *Liquiritiae extractum*—LE, and *Avenae extractum*—AE) were obtained in two successive extraction stages with 50% ethanol, and were dried using lyophilization as described previously [21]. The three extracts were initially evaluated for their toxicity and teratogenic potential on *Daphnia magna* and *Daphnia pulex*. Subsequently, for the activity evaluation assays, we selected concentration ranges based on these preliminary toxicity tests and additional prescreening tests to ensure the measurable effects in the study's specific assays. Thus, for *Betulae extractum* and *Liquiritae extractum*, concentrations ranging from 12.5 to 50 μg/mL were chosen, whereas for *Avenae extractum*, a slightly higher range of 25 to 100 μg/mL was chosen due to its distinct bioactive profile.

A flowchart of the applied methods is illustrated in Figure 1.

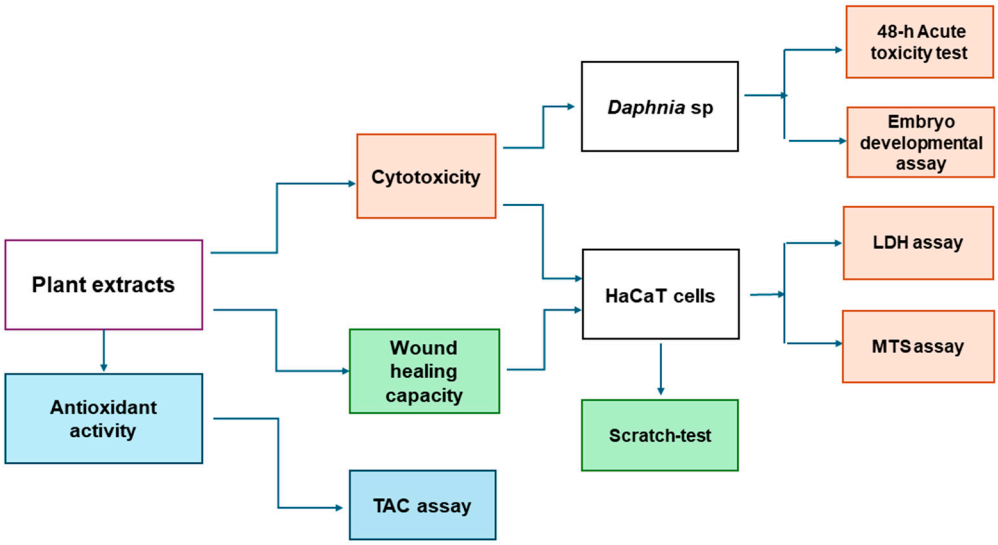


Figure 1. Flowchart of applied methods.

2.4. Cytotoxicity Assay (LDH Assay)

The LDH assay is a colorimetric method of testing cellular cytotoxicity, quantitatively measuring the release of the stable, cytosolic lactate dehydrogenase enzyme by damaged cells. When cell damage occurs, the leakage of cytoplasm into the culture medium increases, and implicitly, the release of LDH increases. The cytotoxicity assay was performed using the CytoTox 96® Non-Radioactive Cytotoxicity Assay kit [27], which measures the conversion of a tetrazolium salt (iodonitro-tetrazolium violet, INT) to a red formazan compound in a 30 min assay, by two coupled enzymatic reactions, catalyzed by LDH and diaphorase. The number of cells lysed is proportional to the red compound formed. A 100% cell lysis positive control was used to determine the maximum LDH present [28,30]. After 24 h of incubation, 25 µL of the supernatant from each well was transferred to another flat-bottomed 96-well plate with 25 µL of reconstituted substrate was added to each well, incubated in the dark and at room temperature for 30 min; subsequently, the reaction was stopped, and the absorbance was read at 490 nm (within a maximum 1 h after stopping the reaction). The test was performed in triplicate.

2.5. Cell Viability Assay (MTS Assay)

MTS assay was performed using the CellTiter 96® AQueous One Solution Cell Proliferation Assay kit [31]. The method is based on a colorimetric method for determining the number of viable using a tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS; Owen's reagent) and an electron coupling reagent (phenazine ethosulfate, PES). MTS is bio-reduced by cells to formazan, a colored product soluble in the culture medium, spectrophotometrically measured at 490 nm directly from culture plates without other preparations. This reduction occurs in the presence of NADPH or NADH produced by dehydrogenase enzymes in metabolically active cells. The formazan produced is directly proportional to the number of living cells. Briefly, assays were performed by adding 20 µL of the CellTiter 96® AQueous One Solution Reagent directly to culture wells, incubating for 2 h, and then recording the absorbance at 490 nm with a 96-well plate reader [28,31]. The test was performed in triplicate.

2.6. Total Antioxidant Capacity Assay (TAC Assay)

The OxiSelect™ Total Antioxidant Capacity (TAC) Assay kit (Cell Biolabs Inc., San Diego, CA, USA) [29] was used to measure the total antioxidant capacity of plant extracts. TAC is based on a SET-type mechanism that reduces copper (II) to copper (I) by antioxidants (e.g., uric acid). Once reduced, the copper I ion reacts with a chromogenic reagent, developing a color with a maximum absorbance at 490 nm. The net absorbance value is compared to the

uric acid standard curve and is proportional to the reducing capacity of the sample. Results are expressed as "μM reducing copper equivalents" (RCE) or "mM uric acid equivalents" (UAE). The cell samples and the working solutions were prepared according to the protocol in the reagent kit [29,32]. The test was performed in triplicate.

2.7. Daphnia Species Acute Toxicity Assay

The bioassays were conducted following a modified protocol based on the established guidelines [33]. Initially, specimens of both *Daphnia magna* and *Daphnia pulex* were selected from parthenogenetic cultures to ensure uniformity in the test population. The assay used 12-well tissue culture plates (Greiner Bio-One, Kremsmünster, Austria), each with 10 daphnids. The extracts were tested at different concentration ranges. The AE and LE extracts were prepared at six concentrations: 1000, 750, 500, 250, 100, and 50 μg/mL, whereas BE extract was tested at concentrations of 330, 250, 165, 50, 30, and 15 μg/mL. All tests were performed in duplicate. The assay conditions were controlled using a climatic chamber (MLR-351H; Sanyo, Osaka, Japan) at 25 °C and 75% relative humidity. Observations for lethality were recorded at 24 and 48 h of exposure. Following the bioassay, the lethal concentrations for 50% of the population (LC_{50}), along with the 95% confidence intervals (95% CI), were calculated using the least square fit method (GraphPad Prism v 5.1 software).

2.8. Daphnia Magna Embryo Developmental Assay

The embryo assay followed the protocols mentioned in the literature [33,34]. Briefly, five embryos were added in wells with selected concentrations of extracts—AE (50 and 500 μg/mL), BE (15 μg/mL), LE (50 μg/mL) (12-well tissue culture plates, Greiner Bio-One). The concentrations were selected based on the findings from the initial toxicity tests. Each concentration was tested in duplicate, and the development was evaluated against an untreated control under the same conditions as those used in the toxicity assays. Microscopic examinations were performed every 24 h using a Euromex bScope to monitor the developmental progress and detect abnormalities in the embryos.

2.9. The "Scratch-Test" on Human Epidermal Keratinocytes

The test involves creating an artificial wound, a "scratch", on a confluent monolayer and monitoring the rate of cell migration by comparing the images captured at the beginning and at set intervals until the cell–cell contacts are restored (the scratch closure). The "scratch" was made with the automatic Autoscratch device from Biotek, followed by the removal of detached cells by repeated washings with phosphate-buffer solution (PBS); after that, the samples to be analyzed are introduced into the cell growth medium. The plates are transferred to the Cytation 5 multimodal cell imaging reader (BioTek, Agilent, Santa Clara, CA, USA) for image acquisition for 24 h in controlled temperature and humidity conditions (37 °C and 5% CO_2). The kinetic analysis of the captured images was performed with the device's imaging software (Gen5 software, version 3.15).

2.10. Statistical Analysis

All data are presented as means ± standard deviations (Mean ± SD) for each prepared sample. Essential conditions for applying statistical tests were evaluated. The normality of the data was assessed using the Shapiro–Wilk test, histograms, and skewness/kurtosis values. Outlier values from the analyzed data sets were excluded if the applied normality tests were met. In order to compute resistant transformation data, an inverse distribution function was used so that they could be normally distributed and subjected to statistical tests. Multiple comparisons to a control (many-to-one comparisons) were performed via Dunnett's test, which computed significant differences at $p < 0.05$ by comparing some treatments with a single control group. All statistical analysis was performed using IBM SPSS Statistics software version 29.0 (IBM Corporation, Chicago, IL, USA). Principal Component Analysis highlighted the correlations between variables. Heatmaps evidenced the

differences between all 3 extracts regarding bioactive compounds and pharmacological properties (XLSTAT 2024.1.0. 1418 by Lumivero—Denver, CO, USA) [35].

3. Results

3.1. Cytotoxicity Assay (LDH Assay)

During the test, as the cell membrane integrity is damaged, the LDH release increases and catalyzes the redox reaction of nicotinamide adenine dinucleotide (NADH) to produce NAD+ (LDH oxidizes lactate to pyruvate), with a reduction in yellow tetrazolium salt to red-colored formazan [20]. The cytotoxic effect of the tested bioactive extracts was plotted as the percentage of cytotoxicity of the sample compared to the LDH-positive control.

Following the analysis of the cytotoxic profiles (Figure 2), for keratinocytes treated with BE, a slight alteration of cellular metabolism was observed, deduced from the increase in the amount of LDH enzyme released into the extracellular environment. The results are plotted as a percent increase in cytotoxicity in a dose-effect manner. The highest percentage of cytotoxicity was 9.63 ± 1.73%, obtained for the 50 µg/mL concentration. Statistically significant differences were reported between all the BE treatment concentrations and the control group cytotoxicity (Table 1) for the same exposure period on normal human keratinocyte cell line (BE 12.5 µg/mL vs. Control group: $p = 0.006$, $p < 0.05$; BE 25 µg/mL vs. Control group: $p = 0.007$, $p < 0.05$; BE 50 µg/mL vs. Control group: $p = 0.011$, $p < 0.05$).

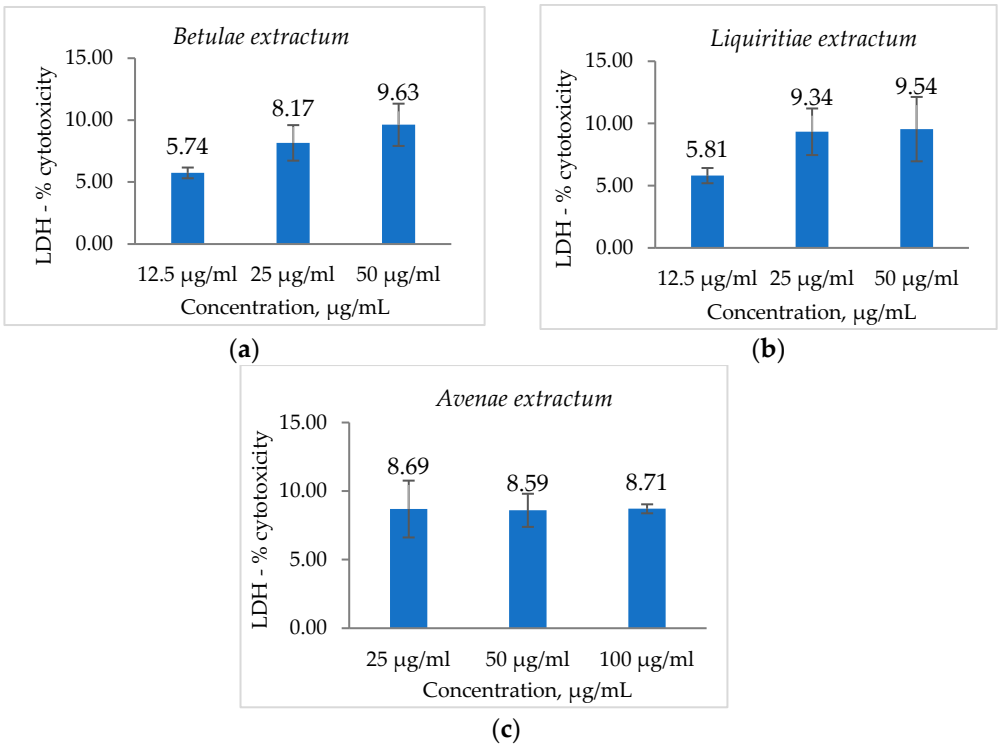


Figure 2. Cytotoxic profile induced using BE (**a**), LE (**b**), and AE (**c**) extracts on normal human keratinocyte cell lines (HaCaT). Data are obtained from three different experiments, and are expressed as percentage cytotoxicity of the sample relative to the LDH positive control. All numerical values are expressed as mean ($n = 3$) ± standard deviation (SD).

Dunnett's test (Table 1) was selected because it is considered the ideal statistical test that compares the hypothesis to a control group. Thus, because we need specific comparisons among means, we selected it to perform and evaluate the differences between the experimental and control groups.

Table 1. Dunnett *t*-test for multiple comparisons between the BE treatment group and the control group (LDH assay).

(I) Treatment_BE	(J) Control_Group	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval
					Upper Bound
BE 12.5 µg/mL	Control_Group	−0.6590 *	0.20869	0.006	−0.2197
BE 25 µg/mL	Control_Group	−0.5989 *	0.19321	0.007	−0.1921
BE 50 µg/mL	Control_Group	−0.6041 *	0.20869	0.011	−0.1648

* The mean difference is significant at the 0.05 level.

In the case of keratinocytes treated with different doses of LE, a slight impairment of the membrane integrity was observed (with increasing enzyme activity in the culture medium), expressed by a maximum percentage value of cytotoxicity of 9.54 ± 2.58%, obtained for the concentration of 50 µg/mL of tested extract.

Substantial differences (Table 2) were also observed between all the selected concentrations of the LE treatment group and the control group (LE 12.5 µg/mL vs. Control group: $p = 0.008$, $p < 0.05$; LE 25 µg/mL vs. Control group: $p = 0.004$, $p < 0.05$; LE 50 µg/mL vs. Control group: $p = 0.019$, $p < 0.05$).

Table 2. Dunnett *t*-test for multiple comparisons between the LE treatment group and the control group (LDH assay).

(I) Treatment_LE	(J) Control_Group	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval
					Upper Bound
LE 12.5 µg/mL	Control_Group	−0.6276 *	0.20630	0.008	−0.1933
LE 25 µg/mL	Control_Group	−0.6464 *	0.19099	0.004	−0.2443
LE 50 µg/mL	Control_Group	−0.5405 *	0.20630	0.019	−0.1062

* The mean difference is significant at the 0.05 level.

For AE, a similar cytotoxic effect was observed for all tested concentrations, the percentage value of the cytotoxicity being in the range 8.69 ± 2.09%–8.71 ± 0.35%, with significant differences between the AE treatment group and the control group (AE 25 µg/mL vs. Control group: $p = 0.007$, $p < 0.05$; AE 50 µg/mL vs. Control group: $p = 0.007$, $p < 0.05$; AE 100 µg/mL vs. Control group: $p = 0.010$, $p < 0.05$). The mean differences were significant at the 0.05 level (Table 3).

Table 3. Dunnett *t*-test for multiple comparisons between the AE treatment group and the control group (LDH assay).

(I) Treatment_AE	(J) Control_Group	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval
					Upper Bound
AE 25 µg/mL	Control_Group	−0.6527 *	0.20893	0.007	−0.2128
AE 50 µg/mL	Control_Group	−0.5972 *	0.19343	0.007	−0.1900
AE 100 µg/mL	Control_Group	−0.6138 *	0.20890	0.010	−0.1740

* The mean difference is significant at the 0.05 level.

From all the results, it can be seen that for BE and LE, the concentrations with the lowest cytotoxicity are 12.5 µg/mL, and for AE is 50 µg/mL (Table 4).

Table 4. The extracts' minimum and maximum cytotoxic doses tested on human keratinocyte cell lines.

Plant Extract	Concentration, µg/mL	% Cytotoxicity
Betulae extractum	12.5	5.74 ± 0.46
	50	9.63 ± 1.73
Liquiritiae extractum	12.5	5.81 ± 0.64
	50	9.54 ± 2.58
Avenae extractum	50	8.59 ± 1.20
	100	8.71 ± 0.35

3.2. Cell Viability Assay (MTS Assay)

The reduction process of MTS tetrazolium compound to soluble, purple formazan dye is controlled by NAD(P)H-dependent oxidoreductase enzymes in the mitochondria of viable cells [20]. The effect of bioactive extracts on cell viability was plotted as the percentage of viability of the sample relative to the corresponding control. In the case of keratinocytes treated with BE, a decrease in cell viability of up to 25% was observed (Figure 3) for the highest concentration tested, namely 50 µg/mL ($p < 0.05$). A similar effect was noted for the cells treated with LE, where cell viability decreased by up to 35% at the 50 µg/mL concentration. Cell viability was slightly affected for cells treated with different concentrations of AE. Its decrease was 8.4% at 25 µg/mL, respectively, and 3.8% at 100 µg/mL. Simultaneously, an appreciable proliferative capacity can be noted for the cells treated with AE at a concentration of 50 µg/mL (Figure 3).

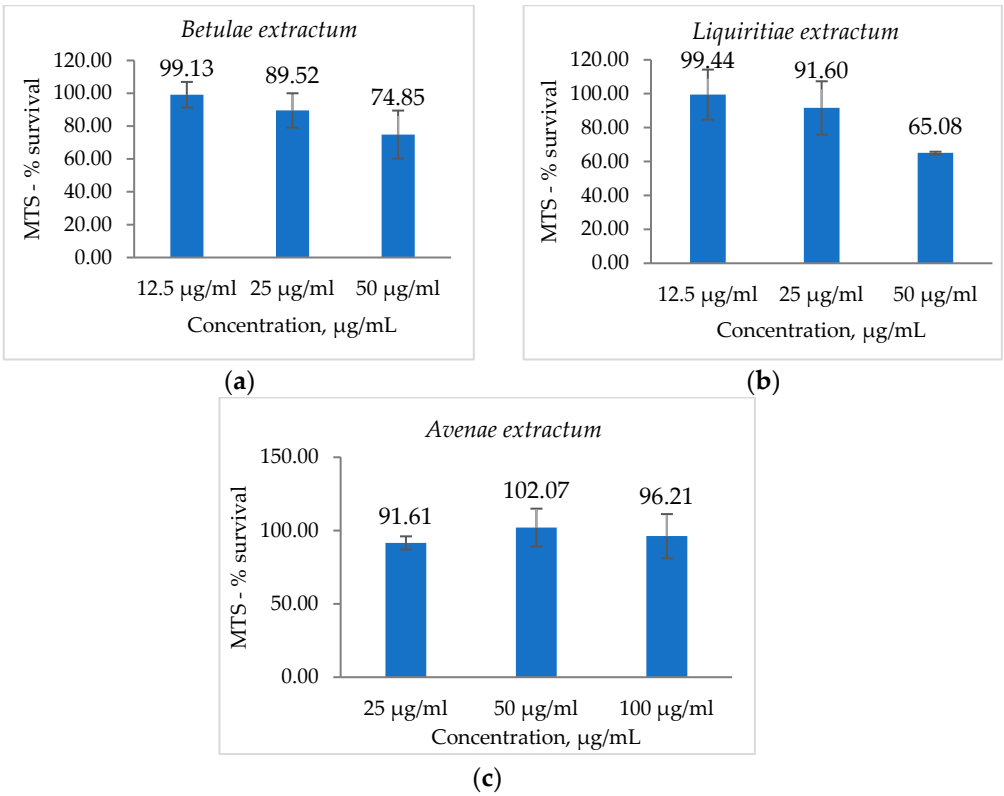


Figure 3. Influence of BE (**a**), LE (**b**), and AE (**c**) extracts on cell viability in the normal human keratinocyte (HaCaT) cell line. Data are obtained from three different experiments and are expressed as the percent viability of the sample relative to the cellular control. All numerical values are expressed as mean ($n = 3$) ± standard deviation (SD).

Statistically significant differences (viability 74.85% vs. Control group, $p = 0.019$, $p < 0.05$) were recorded exclusively at 50 µg/mL concentration for BE extract.

The results highlight that the best proliferative capacity for BE and LE was obtained at the concentration of 12.5 µg/mL, and for AE at the concentration of 50 µg/mL (Table 5), the differences not being statistically significant compared to the control ($p > 0.05$). The recorded values (as a percentage of viability) are correlated with the cytotoxicity values at the following concentrations: the lower the cytotoxicity, the better the proliferative capacity.

Table 5. Proliferative capacity of bioactive extracts tested on normal human keratinocyte line.

Plant Extract	Concentration, µg/mL	% Viability
Betulae extractum	12.5	99.13 ± 7.93
Liquiritiae extractum	12.5	99.44 ± 14.92
Avenae extractum	50	102.07 ± 13.27

Experimental data processed statistically, as well as hypothesis testing, for the LDH assay and MTS assay are illustrated in Supplementary Materials Figures S1–S18.

3.3. Total Antioxidant Capacity Assay (TAC Assay)

Copper-based antioxidant assays are advantageous over iron-based because all classes of antioxidants, including thiols, are detected with marginal radical interference, and because both hydrophilic and lipophilic samples are compatible with this test [36].

The uric acid standard curve obtained based on the dilutions recommended by the kit protocol had a considerable correlation factor, R^2, about 0.9945 (Figure 4).

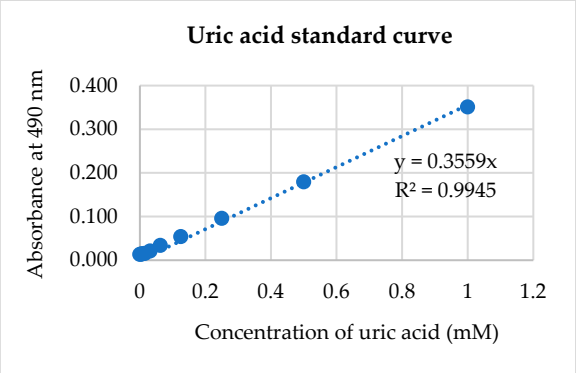


Figure 4. The uric acid standard curve.

For the evaluation of the cellular antioxidant potential, two concentrations were selected for each dry extract based on cytotoxicity results: for BE and LE—12.5 and 25 µg/mL, and for AE—25 and 50 µg/mL. Table 6 registers the values obtained from the triplicate tests compared to a solvent blank (ethanol).

Table 6. The cellular total antioxidant capacity results for the dry extracts.

Plant Extract	Concentration of Extract Solution, µg/mL	mM Uric Acid Equivalent, UAE	µM Reducing Copper Equivalent, RCE
Ethanol	50	0.062 ± 0.005	136.54 ± 9.95
Betulae extractum	12.5	0.068 ± 0.003	149.15 ± 7.89
	25	0.074 ± 0.008	162.55 ± 16.97
Liquiritiae extractum	12.5	0.067 ± 0.005	146.08 ± 11,26
	25	0.072 ± 0.008	158.55 ± 17.60
Avenae extractum	25	0.069 ± 0.003	151.03 ± 6,95
	50	0.071 ± 0.008	155.82 ± 16.84

Experimental data processed statistically, as well as hypothesis testing, for the TAC assay are illustrated in Supplementary Materials Figures S19–S36.

From the results, it can be seen that the cellular antioxidant activities of the dry extracts are higher compared to the control (ethanol). For BE and LE, the concentrations with the highest antioxidant activity were 25 µg/mL (162.55 ± 16.97 for BE and 158.55 ± 17.60 for LE), and for AE it was 50 µg/mL (155.82 ± 16.84), almost similar to the value obtained for the concentration of 25 µg/mL.

Among all three extracts, BE shows higher antioxidant activity than LE and AE, with similar conclusions obtained previously in the chemical assays [21]. The results obtained were statistically significant ($p < 0.05$) for the highest concentration of BE and LE treatment and for both concentrations tested for AE (Tables 7 and 8).

Table 7. Dunnett *t*-test for multiple comparisons between the dry extract treatment and Control group (TAC assay-UAE value).

(I) Treatment	(J) Control_Group	Sig. (*p*-Value)
BE 25 µg/mL	Control_Group	0.020 *
LE 25 µg/mL	Control_Group	0.029 *
AE 25 µg/mL	Control_Group	0.043 *
AE 50 µg/mL	Control_Group	0.001 *

* $p < 0.05$.

Table 8. Dunnett *t*-test for multiple comparisons between the dry extract treatment and Control groups (TAC assay-RCE value).

(I) Treatment	(J) Control_Group	Sig. (*p*-Value)
BE 25 µg/mL	Control_Group	0.019 *
LE 25 µg/mL	Control_Group	0.025 *
AE 25 µg/mL	Control_Group	0.037 *
AE 50 µg/mL	Control_Group	0.001 *

* $p < 0.05$.

3.4. Toxicity Bioassays on Daphnia Species

The lethality curves obtained for the three extracts are presented in Figure 5. The AE extract showed the lowest toxicity among the three extracts. For *Daphnia pulex*, the lethality increased with concentration, but remained moderate, with a value of 45% at the highest concentration (1000 µg/mL) after 48 h. The LC_{50} values are notably higher for AE at 1507 µg/mL and 1152 µg/mL at 24 and 48 h, respectively, indicating a relatively lower toxicity than the other extracts. *Daphnia magna* showed even less sensitivity, with no concentration reaching a 50% lethality rate; thus, the AE was considerably less toxic to this species. LE extract exhibited significantly higher toxicity levels. On *Daphnia magna*, lethality was 100% at the highest concentrations, with lower values of LC_{50} (Table 9). For *Daphnia pulex*, the lethality was moderate at 24 h and high at 48 h. The LC_{50} values obtained and the observed high mortality rates confirm the high toxicity of the LE extract.

BE extract showed a high toxicity level similar to LE. For *Daphnia pulex*, with a progressive increase in lethality with the concentration and duration of exposure (Table 9). On *Daphnia magna*, BE was highly lethal only at the highest concentration tested, with an LC_{50} at 154.1 µg/mL at 24 h, indicating a strong but slightly delayed toxic effect compared to LE. The lethality and LC_{50} comparisons indicate that LE and BE extracts are significantly more toxic to both *Daphnia* species than the AE extract. The lower LC_{50} values associated with LE and BE suggest a higher potency and potential toxicity of these extracts.

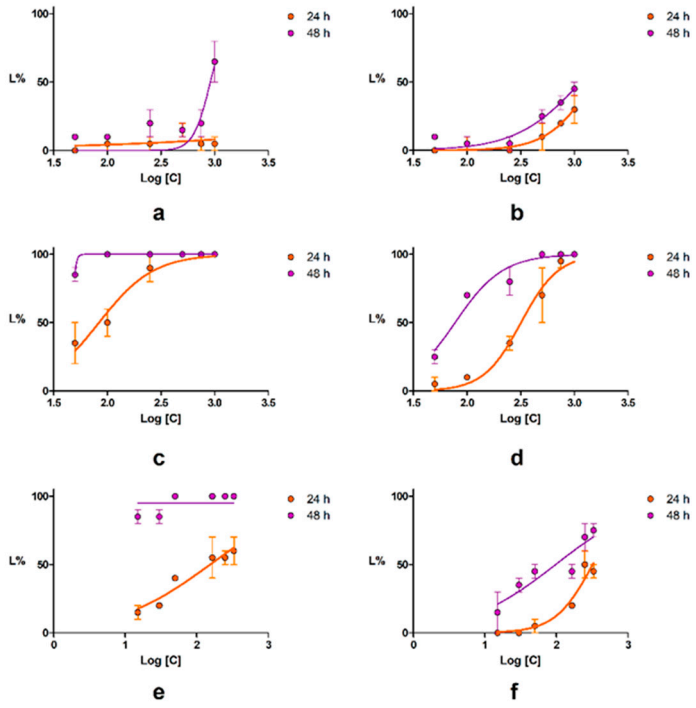


Figure 5. The lethality curves obtained after 48 h exposure of *Daphnia magna* (**a**,**c**,**e**) and *Daphnia pulex* (**b**,**d**,**f**) to the three extracts: *Avenae* extract—(**a**,**b**); *Liquiritiae* extract—(**c**,**d**); and *Betulae* extract—(**e**,**f**). Error bars represent the SE of two replicates.

Table 9. Results of *Daphnia* species lethality bioassays.

Daphnia Species	Plant Extract	LC_{50} (µg/mL)		95% CI	
		24 h	48 h	24 h	48 h
Daphnia pulex	AE	1507	1152	906.3–2506	853.6–1555
	LE	318.5	77.47	254.5–398.6	63.31–94.80
	BE	321.2	97.58	252.0–409.5	57.83–164.60
Daphnia magna	AE	NC *	NC *	NC *	NC *
	LE	83.74	47.15	63.06–111.2	NC **
	BE	154.1	NC **	96.28–246.7	NC **

NC *—the lethality was below 50%. NC **—the lethality was above 90%.

3.5. Daphnia Magna Embryo Developmental Assay

In the embryo test assessing the effects of AE, BE, and LE extracts on *Daphnia magna* embryos, the results at lower concentrations (50 µg/mL for AE and LE and 15 µg/mL for BE) showed development and morphology that were similar to those observed in the control group. At these concentrations, the formation of the compound eye, antennae, rostrum, post-abdominal claw, and ocellus developed normally (Figure 6a–j). Moreover, the mobility of the young daphnids at 48 h was comparable to that of the untreated control, indicating no adverse effects at these exposure levels.

Based on the lethality results, the AE extract was tested additionally at a 500 µg/mL concentration. All embryos exposed to this concentration remained undeveloped, indicating the total inhibition of developmental processes. This result indicates that although the extract is non-toxic to young daphnids at high concentrations, it can significantly affect the embryo's development. Our results revealed that while lower concentrations of these extracts appear safe and do not interfere with normal embryonic development, the high concentration of AE extract poses significant developmental risks. The lack of any developmental retardation at lower concentrations for all tested extracts suggests that the lower concentrations could be considered non-teratogenic and safe for these species.

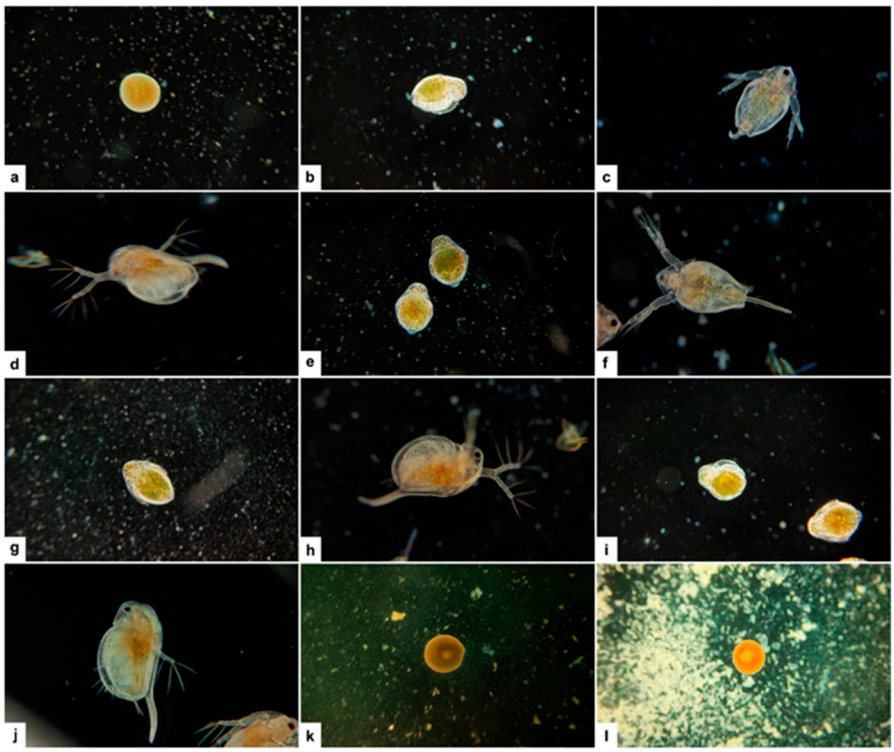


Figure 6. *Daphnia magna* embryo test: (**a**)—embryos at 0 h; (**b**)—intermediary larval stage untreated at 24 h; (**c**,**d**)—young daphnid untreated at 48 h; (**e**)—intermediary larval stage treated with 50 µg/mL LE extract at 24 h; (**f**)—young daphnid treated with 50 µg/mL LE extract at 48 h; (**g**)—intermediary larval stage treated with 15 µg/mL BE extract at 24 h; (**h**)—young daphnid treated with 15 µg/mL BE extract at 48 h; (**i**)—intermediary larval stage treated with 50 µg/mL AE extract at 24 h; (**j**)—young daphnid treated with 50 µg/mL AE extract at 48 h; and (**k**,**l**)—undeveloped embryos treated with 500 µg/mL AE extract at 24 and 48 h.

3.6. The "Scratch-Test" on Human Epidermal Keratinocytes

To evaluate the in vitro cell migration process, the experimental model applied to the HaCaT cell line after 24–48 h for adhesion and cell monolayer formation includes two series, as follows: (1) a series of unstimulated cells, maintained in culture in contact with the tested extracts for 24 h; (2) a series of cells maintained in culture in contact with the tested extracts, simultaneously with the mimicking of non-specific inflammation (addition of Tumor necrosis factor-α (TNF-α) 15 ng/mL), associated with oxidative stress (addition of Phorbol myristate acetate (PMA) 0.1 µM), for 24 h.

The tested concentrations of the dry extracts were lower than the maximum toxicity doses resulting from the cytotoxicity assays. The average value of the wound confluence percentage is represented graphically as mean ± standard deviation (SD) for each sample concentration at each selected time.

The values of the kinetic cell-covered area (Object Sum Area) generated using the Gen5 software were used to represent important parameters in the wound healing process. First, the wound width (average width of the cell-free area per time point—µm) was calculated, and its value was further used to estimate wound confluence (percentage of initial wound area covered by migrating cells over time—%) and maximum wound healing rate—$\mu m^2/h$. The results are presented in Figures 7–9.

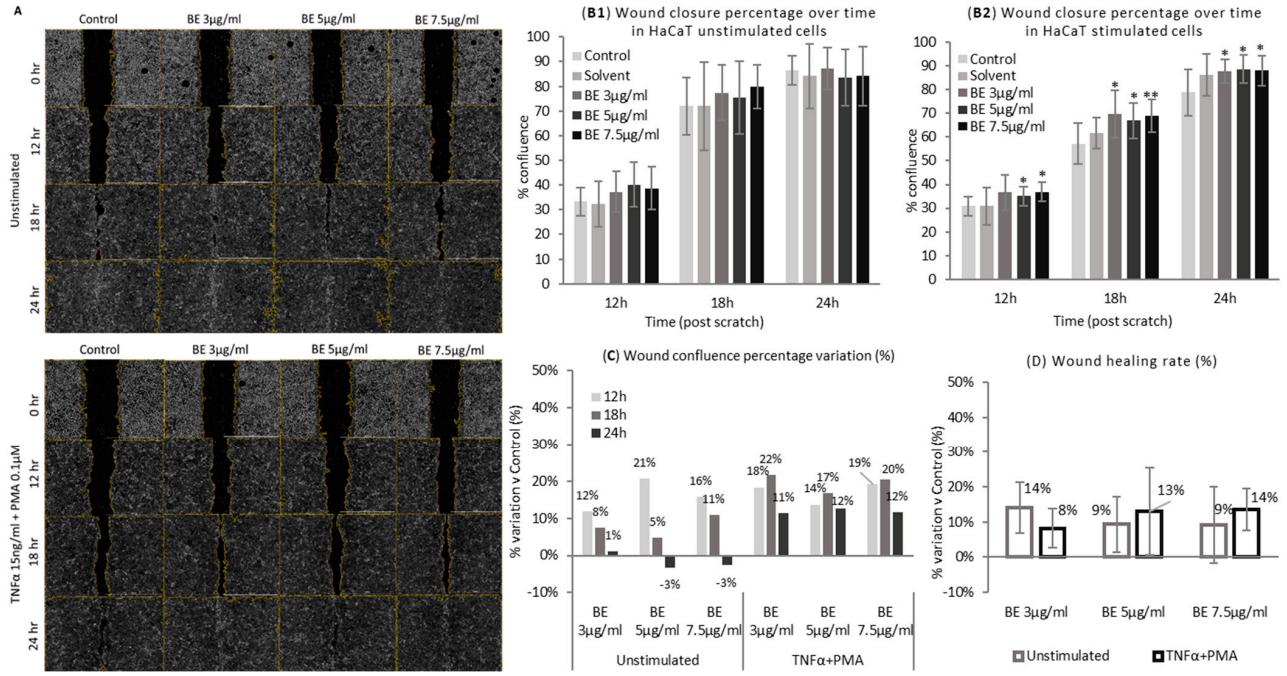


Figure 7. (**A**) Representative images from the in vitro wound healing test illustrating cell migration on the wound site (area marked with yellow) were acquired for 24 h using the 4x magnification objective in bright-field high-contrast mode (scale bar = 1000 µm). The wound healing process evaluation in the presence of BE using image-based cell analysis: the evolution of the wound confluence degree after 12 h and 18 h, respectively, and 24 h in cells treated without stimulation (**B1**), and pro-inflammatory stimulated with TNF-α 15 ng/mL and PMA 0.1 µM (**B2**). Calculation of the variation percentage for the wound confluence degree (**C**) and the wound healing rate (**D**) compared to the untreated cell control. All numerical values are represented as mean (n = 3) ± standard deviation (SD); (* $p < 0.05$; ** $p < 0.01$).

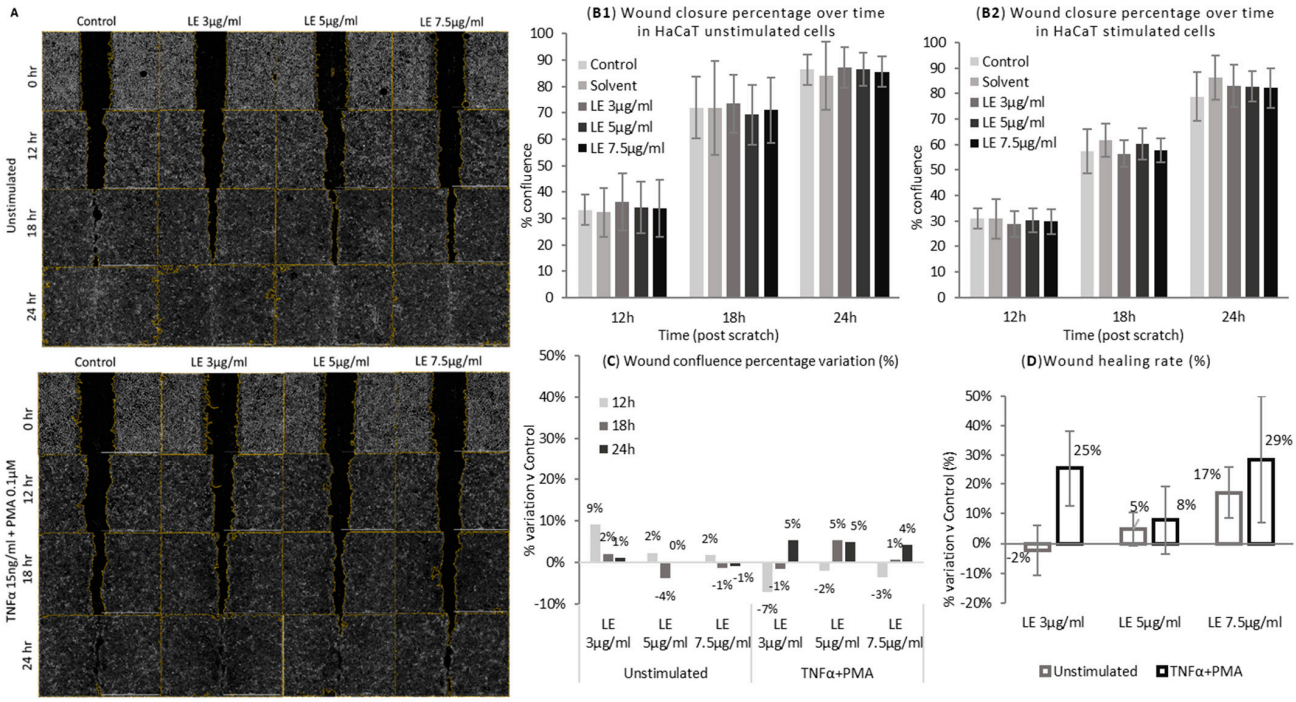


Figure 8. (**A**) Representative images from the in vitro wound healing test illustrating cell migration on the wound site (area marked with yellow) were acquired for 24 h using the 4× magnification

objective in bright-field high-contrast mode (scale bar = 1000 µm). The wound healing process evaluation in the presence of LE, using image-based cell analysis: the evolution of the wound confluence degree after 12 h and 18 h, respectively, and 24 h in cells treated without stimulation (**B1**), and pro-inflammatory stimulated with TNF-α 15 ng/mL and PMA 0.1 µM (**B2**). Calculation of the variation percentage for the wound confluence degree (**C**) and the wound healing rate (**D**) compared to the untreated cell control. All numerical values are represented as mean (n = 3) ± standard deviation (SD); ($p > 0.05$).

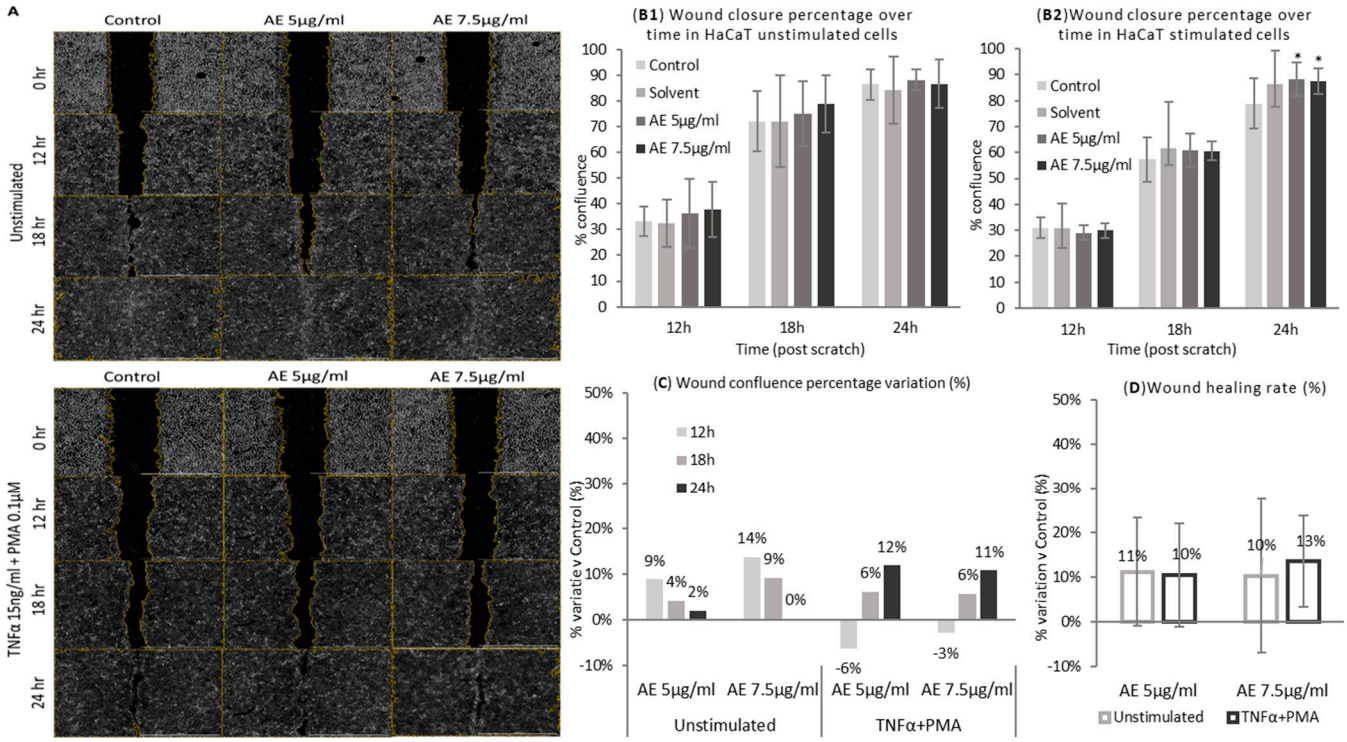


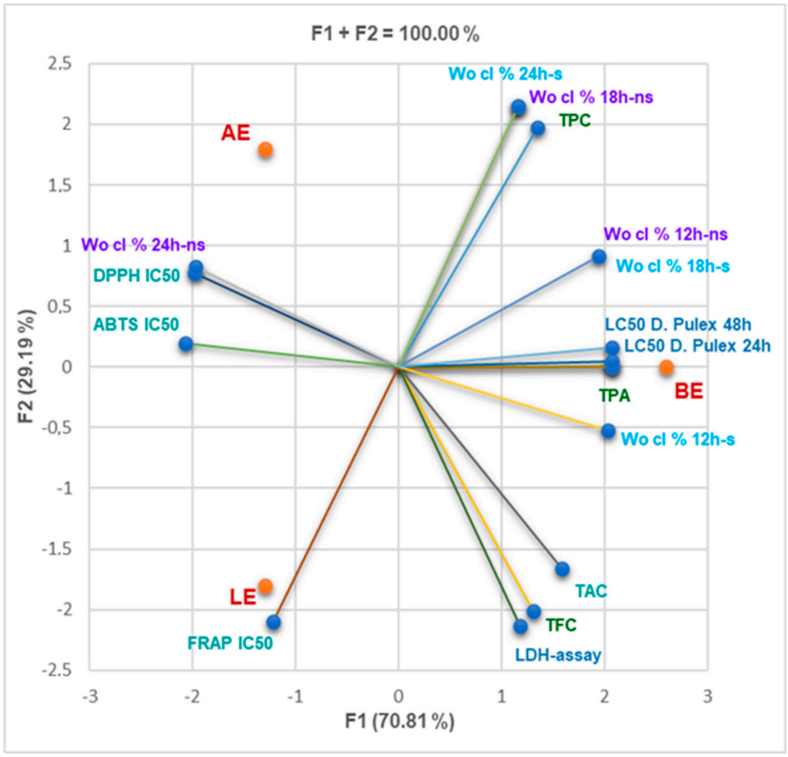
Figure 9. (**A**) Representative images from the in vitro wound healing test illustrating cell migration on the wound site (area marked with yellow). The images were acquired for 24 h using the 4x magnification objective in bright-field high-contrast mode (scale bar = 1000 µm). The wound healing process evaluation in the presence of AE using image-based cell analysis: the evolution of the wound confluence degree after 12 h, and 18 h, respectively, and 24 h in cells treated without stimulation (**B1**), and pro-inflammatory stimulated with TNF-α 15 ng/mL and PMA 0.1 µM (**B2**). Calculation of the variation percentage for the wound confluence degree (**C**) and the wound healing rate (**D**) compared to the untreated cell control. All numerical values are represented as mean (n = 3) ± standard deviation (SD); ($p > 0.05$; * $p < 0.05$).

Following the analysis of the obtained data from the in vitro wound healing assay, for both types of keratinocytes, unstimulated and pro-inflammatory stimulated (TNF-α 15 ng/mL and PMA 0.1 µM), after the treatment with *Betulae extractum*, the percentage of wound confluence reached 87–90% after 24 h. After 12 h, it can be seen that BE increased wound confluence by up to 20% compared to control for both stimulated and unstimulated cells. This trend persists over time, reaching a maximum increase in the percentage of wound coverage of 22% at 18 h post-scratch for the stimulated cells treated with BE 3 µg/mL. By the end of the monitoring interval, only for stimulated cells, this increase in the percentage of cellular migration was maintained by up to 10% compared to the corresponding control. Regarding the wound healing rate, a positive effect of treatment with BE extract was observed, causing an increase of up to 14% compared to the untreated control for both types of cells, pro-inflammatory stimulated and unstimulated.

After the treatment with *Liquiritiae extractum*, for both types of cell cultures, without stimulation and pro-inflammatory stimulated with TNF-α 15 ng/mL and PMA 0.1 μM, the percentage of wound confluence reached 87% after 24 h without a significant difference compared to cell control. However, regarding the wound healing rate, a positive effect of the treatment with LE was observed at the highest applied dose (LE 7.5 μg/mL), causing an increase of up to 17% compared to the untreated control for the unstimulated cells, respectively, and by up to 29% for the pro-inflammatory stimulated cells.

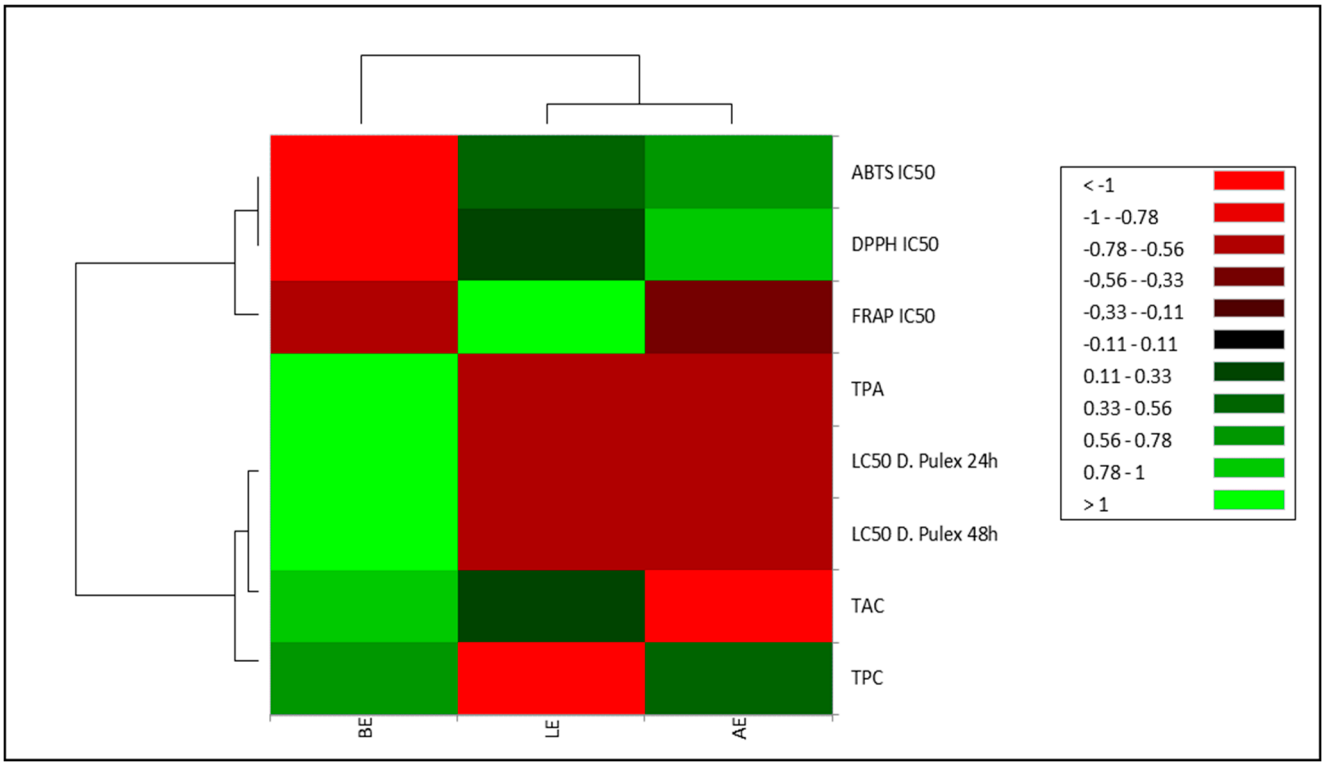
For unstimulated and pro-inflammatory stimulated keratinocytes treated with *Avenae extractum*, the percentage of wound confluence reached 87% after 24 h. After 12 h post-scratch, the AE increased wound confluence by up to 14% in unstimulated cells and 9% in pro-inflammatory stimulated cells, compared to untreated control. By the end of the monitoring interval, only in the case of stimulated cells, this increase in the percentage of cell migration was maintained by up to 12% compared to the corresponding control. The AE of 7.5 μg/mL increased the wound healing rate by up to 10% compared to the untreated control for unstimulated cells, respectively, and by up to 13% in pro-inflammatory stimulated cells.

The correlations between bioactive compounds previously quantified in each extract, antioxidant potential evaluated through DPPH, ABTS, and FRAP assays [21], and the pharmacological properties investigated in the present study are illustrated in Figure 10A. The differences between all three extracts are evidenced in Figure 10B.



(**A**)

Figure 10. *Cont.*



(**B**)

Figure 10. (**A**) Correlations between bioactive constituents and pharmacological potential of all three extracts. (**B**) Hierarchical clusters and heat map analysis. BE = *Betulae cortex* extract; LE = *Liquiritiae radix* extract; AE = *Avenae herba* extract; IC50 = median inhibitory concentration of free radical generation in DPPH, ABTS, and FRAP assays; LC_{50} = median lethal concentration on *D. pulex*; TAC = total antioxidant capacity; Wo cl % = wound closure percentage; 12, 18, 24 = measuring period; ns = unstimulated HaCaT cells; s = pro-inflammatory stimulated HaCaT cells; LDH assay = cytotoxic capacity evaluation assay. Where the results are expressed as percentages: TPA = Total phenolic acids, TPC = total polyphenols content, and TFC = total flavonoid content.

The correlation biplot from Figure 10A is displayed on the first two axes; the linked parameters ensure 100% variability. The total phenolic acid content is substantially correlated with LC_{50} in *D. pulex*; therefore, the lethal effects are inversely proportional to TPA concentration ($r = 0.999$, $p < 0.05$). TPA is also strongly correlated with wound closure percentage on pro-inflammatory stimulated HaCaT cells measured after 18 h ($r = 0.998$, $p < 0.05$) and 12 h ($r = 0.979$, $p > 0.05$) and on unstimulated ones after 12 h ($r = 0.936$, $p > 0.05$). TPA shows a significant negative correlation with ABTS IC_{50} ($r = -0.997$, $p < 0.05$) and DPPH IC_{50} ($r = -0.955$, $p > 0.05$. TPA is moderately correlated with TAC ($r = 0.766$, $p > 0.05$), while TFC is remarkably correlated ($r = 0.982$, $p > 0.05$). TFC is strongly correlated with the cytotoxic effects measured using an LDH assay ($r = 0.997$, $p > 0.05$); therefore, high flavonoid content leads to high cytotoxicity due to the dual redox behavior (antioxidant/prooxidant) of phenolic compounds. TPC displays a significant negative correlation with FRAP IC_{50} ($r = -0.997$, $p < 0.05$); the antioxidant potential increases directly proportional to TPC. DPPH IC_{50} and ABTS IC_{50} strongly correlate negatively with LC_{50} on *D. pulex* and wound closure% on stimulated HaCaT cells ($r < -0.900$, $p > 0.05$). Therefore, *D. pulex* mortality is based on pro-oxidant potential, and antioxidant capacity promotes wound healing. Moreover, Figure 10A shows the place of each, conditioned by all variable parameters.

4. Discussion

This current work evaluated the cytotoxicity, cellular antioxidant potential, and capacity of stimulating keratinocyte migration of three extracts (*Betulae cortex* extract—BE, *Liquiritiae radix* extract—LE, and *Avenae herba* extract—AE). The cytotoxicity was tested in vitro on human normal keratinocytes (HaCaT) cell lines and in vivo on *Daphnia* species. Cell toxicity testing in multiple models is considered necessary experimentally to observe possible differences in results obtained under distinct culture and test conditions. The principle of the cytotoxicity assay is based on lactate dehydrogenase (LDH), a cytosolic enzyme present in all cells, which remains in the cytoplasm under normal physiological conditions of the plasma membrane. In vitro LDH release provides a precise way to measure cell membrane integrity and, by implication, cell viability. Cell destruction is inevitable due to the loss of the cell's ability to maintain and provide energy for the metabolic activity of cell function and growth [18]. The results highlight that BE and LE are more cytotoxic than AE. Also, there is a dose-dependent relationship, especially for BE, for which the highest cytotoxicity was obtained at 50 μg/mL and the lowest at 12.5 μg/mL. Almost similar results were obtained for LE, the lowest toxicity associated with the 12.5 μg/mL concentration. The results were almost identical for the other two concentrations, 25 and 50 μg/mL. For AE, there was no dose-dependent relationship for all three tested concentrations (25, 50, and 100 μg/mL), with very close values. However, the cytotoxic effect was significant compared to the control ($p < 0.05$) for all concentrations tested for BE, LE, and AE. Thus, the cytotoxicity was much less in the case of AE because it induced changes at a higher concentration than the other analyzed extracts (BE and LE), which were active at lower doses.

Estimation of metabolic activity is based on mitochondrial activity. For this reason, the MTS assay is used in cell viability and proliferation studies. The conversion of MTS to water-soluble formazan occurs under the action of enzymes (dehydrogenases) found in metabolically active cells. Treatment of cells with MTS allows for the assessment of oxidative metabolism and the response of a cell population to external factors that may have a positive or negative effect on cell life in culture [18]. Thus, the results for the viability assay are in an inverse relationship with LDH assay results: the lower the cytotoxicity, the better the proliferative capacity. The dose-dependent relationship observations remain similar for this assay as for the cytotoxicity test. The MTS assay revealed an appreciable proliferative capacity illustrated for AE 50 μg/mL and for BE and LE at the concentration of 12.5 μg/mL, the cell viability of keratinocytes being significantly affected only by BE 50 μg/mL and LE 50 μg/mL ($p < 0.05$).

In vivo cytotoxicity studies on small freshwater shellfish *Daphnia magna* (daphnids) are often used in determining the toxicity of drugs, plant extracts, various bioactive phytochemicals, and nanomaterials [37]. Due to its high sensitivity to toxic substances, *Daphnia* species have been used for 40 years in ecotoxicology as a standardized test organism (in standardized protocols for chemical toxicity testing, such as the OECD 202 (Acute toxicity) and 211 (Reproduction) tests and the EPA testing of chemicals) [37,38]. The toxicity of a compound is usually determined either as mean lethal concentration (LC_{50}) or as a change in an organism's reproductive capacity. These tests are simple, reproducible, and are particularly valued for their alignment with ethical research practices while providing critical insights into the environmental impact of substances on aquatic organisms [33,39]. Moreover, the *Daphnia* assay is especially suitable for evaluating plant extracts' toxicity. By this method, a rapid assessment of the effects of different concentrations of plant extracts on the survival and health of *Daphnia* species can be performed, obtaining valuable results for further pharmaco-toxicological and ecological testing [40].

The results from in vivo toxicity assays using the three extracts on *Daphnia magna* and *Daphnia pulex* showed different responses between the two *Daphnia* species to the extracts' species-specific susceptibilities. *Daphnia pulex* displayed increased sensitivity to the extracts compared to *Daphnia magna*. The lethality data indicated that while AE extract exhibited relatively low toxicity, for LE and BE extracts, both species showed high toxicity levels,

especially at higher concentrations, suggesting potent bioactive compounds within these extracts. The embryo test results for *Daphnia magna* showed that lower concentrations (50 µg/mL for AE and LE, 15 µg/mL for BE) are non-teratogenic for this species. However, the complete developmental arrest observed in embryos exposed to 500 µg/mL of AE indicates a critical toxicity threshold.

The in vitro "scratch test" represents an essential tool for evaluating the skin restructuring process in preclinical studies. It contributes to a better understanding of the mechanisms that regulate cell migration, and allows the obtaining of preliminary results regarding the effects of cell migration in different experimental conditions. It is a standard test method for liquid samples [41]. Performing this test allows us to evaluate the intercellular interactions and cell migration due to their interaction with the extracellular matrix (ECM), being able to mimic cell migration in vivo [42,43]; thus, it represents an integral part of the preclinical development, with relevance for further in vivo studies. The tissue remodeling process is dynamic and complex. It involves several stages, including the inflammatory stage, during which a series of growth factors and pro-inflammatory cytokines are released at the wound site. To mimic these acute inflammation conditions, normal keratinocytes were stimulated with Tumor necrosis factor-α (TNF-α), which is a pro-inflammatory cytokine produced by macrophages/monocytes during the acute inflammation phase, and is rapidly released and initiates inflammation in the wound tissues [44]), associated with a pro-oxidant agent (Phorbol myristate acetate (PMA), which activates neutrophils—the first circulating inflammatory cells that move to the wound site—thus being a promoter of acute inflammation [45]).

Our study results confirm these plant extracts' effectiveness in stimulating skin cell migration, highlighting a potential anti-inflammatory effect by increasing the rate of cell migration in conditions of induced inflammation associated with oxidative stress. Among the concentrations tested for the three selected extracts, with a potential contribution to the wound healing process by (1) maintaining the homeostasis of the epidermal extracellular matrix, (2) restoring the extracellular matrix, (3) significantly increasing the wound healing rate, stand out: BE 3 µg/mL, LE 7.5 µg/mL, and AE 7.5 µg/mL.

From the cellular antioxidant assay, as an essential mechanism for the re-epithelialization process, it can be seen that BE has the most significant potential from the three dry extracts tested, and the least potential is associated with AE. These results connect to those obtained from chemical tests (DPPH, ABTS, and FRAP) previously described [21].

Regarding the effectiveness of tested extracts in stimulating the rate of cell migration, the literature describes birch bark as a traditional medicinal remedy known to accelerate the healing process of wounds, having excellent antioxidant, anti-inflammatory, and antibacterial properties. Responsible for these actions are the triterpenes, for which there are data in the literature on the mechanisms by which they sustain these effects. Betulin is the main compound that triggers the inflammatory cascade necessary to stimulate cell proliferation, and lupeol and erythrodiol influence cell migration by rearranging the actin cytoskeleton. The mixture of triterpene compounds can induce the expression of some differentiation markers (keratin 1, keratin 10, involucrin, filaggrin) and increase the calcium influx, which is essential in proliferation [46,47].

For licorice root, the flavonoid, triterpene, and saccharide content are responsible for the anti-inflammatory and antioxidant actions and the stimulation of re-epithelialization. For several studies on rats, applying alcoholic and hydroalcoholic extracts from licorice root has demonstrated their ability to modulate physiological processes in skin injuries by stimulating cell migration through the antiradical, anti-inflammatory, and antibacterial effects [48,49].

For *Avena sativa*, positive results have been identified in the literature regarding the stimulation of re-epithelialization using ethanolic extracts from the aerial parts of oats after flowering due to the content of beta-glucan, flavones, and avenanthramides [50].

Concerning *Avenae herba* extract, as far as we know, there is no evidence in the literature about the healing potential of the herb harvested before flowering. In our study, the

hydroalcoholic extract of oat, at a concentration of 7.5 µg/mL, induced an increase in the wound healing rate by up to 13% in pro-inflammatory stimulated cells and an increase in wound confluence by up to 9% after a 24 h treatment compared to the corresponding control, highlighting a good anti-inflammatory and re-epithelialization activity.

All significant statistical results obtained with the correlations between bioactive constituents and pharmacological potential of all three extracts, hierarchical clusters, and heat map analysis validate the possible clinical relevance of our determinations. By applying Dunnett's statistical procedure to such randomized one-way designs, we could outline the therapeutic efficacy profile for the treatments that give results superior to the control. In addition, comparisons of multiple treatment groups with a control (or placebo) group using powerful and significant statistical methods are very relevant to the phytopharmacology area and meet Good Statistical Practice criteria in pharmacology and biomedical experiments. Furthermore, the good comparability of the assays could be explained by the mechanism of action of the analyzed plant extracts, which can lead to an effective re-epithelialization and proliferative capacity of damaged skin cells as a result of the potent antioxidant effect at the cellular level with a protective role.

5. Conclusions

Despite the fact that the three extracts demonstrated toxic effects at higher concentrations, in vitro assays on HaCaT cells and in vivo tests on *Daphnia* species suggest that they can be considered safe at lower levels (12.5 µg/mL for *Betulae extractum* and *Liquiritiae extractum*, and 50 µg/mL for *Avenae extractum*). Concerning the re-epithelialization process, the results revealed that all three dry extracts are effective in stimulating skin cell migration, also highlighting a potential anti-inflammatory effect by increasing the cell migration rate in conditions of induced inflammation associated with oxidative stress. Among the tested concentrations with a potential contribution to wound healing, the following standout: birch bark extract 3 µg/mL, licorice root extract 7.5 µg/mL, and oat herb (harvested before flowering) extract 7.5 µg/mL.

The results are promising, but due to the methods' limitations, our study represents a starting point for further extracts of long-term safety and efficacy research within the development of personalized topical products for xerosis treatment based on the characteristic symptoms.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/cosmetics11040124/s1. Figure S1: Histogram MTS–AE; Figure S2: Histogram MTS–BE; Figure S3: Histogram MTS–LE; Figure S4: Normal Q-Q Plot MTS–AE; Figure S5: Normal Q-Q Plot MTS–BE; Figure S6: Normal Q-Q Plot MTS–LE; Figure S7: Estimated Means MTS–AE; Figure S8: Estimated Means MTS–BE; Figure S9: Estimated Means MTS–LE; Figure S10: Estimated Means LDH–BE; Figure S11: Estimated Means LDH–LE; Figure S12: Estimated Means LDH–AE; Figure S13: Histogram LDH–LE; Figure S14: Normal Q-Q Plot LDH–LE; Figure S15: Histogram LDH–BE; Figure S16: Normal Q-Q Plot LDH–BE; Figure S17: Histogram LDH–AE; Figure S18: Normal Q-Q Plot LDH–AE; Figure S19: Histogram TAC_UAE_BE; Figure S20: Normal Q-Q Plot TAC_UAE_BE; Figure S21: Estimated Means TAC_UAE_BE; Figure S22: Histogram TAC_UAE_LE; Figure S23: Normal Q-Q Plot TAC_UAE_LE; Figure S24: Estimated Means TAC_UAE_LE; Figure S25: Histogram TAC_UAE_AE; Figure S26: Normal Q-Q Plot TAC_UAE_AE; Figure S27: Estimated Means TAC_UAE_AE; Figure S28: Histogram TAC_RCE_BE; Figure S29: Normal Q-Q Plot TAC_RCE_BE; Figure S30: Estimated Means TAC_RCE_BE; Figure S31: Histogram TAC_RCE_LE; Figure S32: Normal Q-Q Plot TAC_RCE_LE; Figure S33: Estimated Means TAC_RCE_LE; Figure S34: Histogram TAC_RCE_AE; Figure S35: Normal Q-Q Plot TAC_RCE_AE; Figure S36: Estimated Means TAC_RCE_AE.

Author Contributions: Conceptualization, A.G., M.L.T., C.M.N., A.T., L.P., E.A.L., O.T.O., V.P., R.B. and C.E.G.; methodology, A.G., M.L.T., C.M.N., A.T., L.P., E.A.L., O.T.O., V.P., T.D.B., L.E.D., R.B. and C.E.G.; software, A.G., M.L.T., C.M.N., A.T., L.P. and V.P.; validation, A.G., M.L.T., C.M.N., A.T., L.P., E.A.L., O.T.O., V.P., R.B. and C.E.G.; formal analysis, A.G., M.L.T., C.M.N., A.T., L.P., E.A.L., O.T.O., V.P., T.D.B., L.E.D., R.B. and C.E.G.; investigation, A.G., M.L.T., C.M.N., A.T., L.P., E.A.L., O.T.O., V.P.,

R.B. and C.E.G.; resources, A.G., M.L.T., C.M.N., A.T., L.P., E.A.L., O.T.O., V.P., R.B. and C.E.G.; data curation, A.G., M.L.T., C.M.N., A.T., L.P., E.A.L., O.T.O., V.P., R.B. and C.E.G.; writing—original draft preparation, A.G., M.L.T., C.M.N., A.T., L.P., E.A.L., O.T.O., V.P., R.B. and C.E.G.; writing—review and editing, A.G., M.L.T., C.M.N., A.T., L.P., E.A.L., O.T.O., V.P., T.D.B., L.E.D., R.B. and C.E.G.; visualization, A.G., M.L.T., C.M.N., A.T., L.P., E.A.L., O.T.O., V.P., T.D.B., L.E.D., R.B. and C.E.G.; supervision, A.G., M.L.T., C.M.N., A.T., L.P., E.A.L., O.T.O., V.P., T.D.B., L.E.D., R.B. and C.E.G.; project administration, A.G., M.L.T., C.M.N., A.T., L.P., E.A.L., O.T.O., V.P., R.B. and C.E.G.; funding acquisition, A.G., M.L.T., C.M.N., A.T., L.P., E.A.L., O.T.O., V.P., R.B. and C.E.G. All authors have read and agreed to the published version of the manuscript.

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