UNIVERSITY OF MEDICINE AND PHARMACY
„CAROL DAVILA", BUCHAREST
DOCTORAL SCHOOL
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APLICABILITY OF NATURAL EXTRACTS IN THE PREVENTION AND TREATMENT OF XEROSIS

Ph.D. THESIS SUMMARY

PhD Supervisor:

PROF. UNIV. DR. GÎRD CERASELA-ELENA

PhD Student:

FARM. BICU (GHICA) ADELINA

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INTRODUCTION

The skin tissue represents the barrier that protects the body from external factors, being involved in maintaining homeostasis, in thermoregulation, photoprotection and in defense against microorganisms and harmful chemicals, being, at the same time, the first immunological defense. The water content of the skin plays a crucial role, affecting the physical and mechanical properties of the stratum corneum and the enzymatic activity involved in the processes of keratinocyte proliferation and differentiation, as well as in normal corneocyte desquamation. Alteration of the skin tissue barrier function basically represents the alteration of the stratum corneum integrity, with important morphological and functional disturbances, resulting in xerosis occurance. *Xerosis cutis* is one of the most common skin conditions seen by dermatologists and general practitioners in routine clinical practice. It represents a psycho-social factor with an important impact and, at the same time, a risk factor for the development of atopic or allergic dermatitis, as well as other skin diseases. Epidemiologically, research has shown a worldwide prevalence between 29% and 85%, especially in people over 60 years old and, in particular, also associated with pre-existing medical conditions or as a result of certain medications treatments. The therapeutic approach for advanced xerosis stages involves a combined treatment, an association of moisturizing products with those containing antibiotics, antihistamines and corticosteroids, in order to restore the skin homeostasis. However, most drug treatments are limited to topical administration, as in the case of anti-inflammatory drugs, which in long-term use decrease the percentage of collagen, causing skin atrophy. Because of these risks, natural plant molecules remain current topics for new research. Plant extracts possess a long-term valuable contribution to the prevention and treatment of skin pathologies, including xerosis, by developing antioxidant, anti-inflammatory, antimicrobial, moisturizing, re-epithelizing, photoprotective effects, etc., and also by the ability to modulate skin functions, in order to correct imbalances. The main objective of current therapies is to adapt the treatment to the abnormalities manifested by the generally recognized symptoms, classified into the four stages of xerosis: mild (tendency to dryness), moderate (mild dryness), severe (moderate dryness) and very severe (severe dryness, inflammation).

In this context, the aim of this research is the formulation of three semisolid topical products intended for the personalized approach of the xerosis stages - mild, moderate and severe, based on plant phytocomplexes with therapeutic potential, incorporated in matrices obtained from carefully selected, non-irritating and biocompatible ingredients, in order to

prevent and treat the characteristic symptomatology, by correcting the existing hydro-lipid deficits and disturbances occurring in the physiological processes at the skin level.

The research objectives are: the identification of plant products with applicability in the prevention and treatment of xerosis, as sources of bioactive compounds with relevant activity for the targeted pathology (antioxidant, anti-inflammatory, antimicrobial, regenerative), as well as establishing the extraction methodology, in order to obtain extractive solutions with a complex composition, enriched in active principles of therapeutic interest; obtaining dry plant extracts through optimized processes of concentration in the rotaevaporator and drying by lyophilization, and their characterization by performing a phytochemical screening, in order to identify and quantify the constituent bioactive compounds, through spectrophotometric, chromatographic (GC-MS, HPTLC) and spectrometric (FT-ICR-MS and FT-IR) analyses; defining the cytotoxicity profile of the extracts by *in vitro* methods (cell cytotoxicity assay and cell viability assay on standardized normal human keratinocytes cell line (HaCaT) – representative cells of the epidermis, the skin layer affected in xerosis) and *in vivo* (acute toxicity assay on *Daphnia* species and teratogenic potential on *Daphnia magna* embryos); the *in vitro* evaluation of the extracts antiradical activity (DPPH, ABTS and FRAP methods) and of the total antioxidant capacity (TAC) on standardized normal human keratinocytes cell line (HaCaT), as well as the evaluation of the phytocompounds ability to modulate the intrinsic systems involved in the skin natural antioxidant defence, through molecular docking studies; the *in vitro* assessment of the extracts impact on cell migration rate in the re-epithelialization process, as well as the anti-inflammatory potential, by performing a "scratch test" on keratinocyte grown under normal conditions (without stimulation), and under conditions mimicking non-specific inflammation (stimulation with tumor necrosis factor – TNF-α), associated with oxidative stress (stimulation with phorbol myristate acetate – PMA); establishing the therapeutic directions of the extracts regarding the personalized approach of the xerosis stages, in direct correlation to the identified phytocomplexes and the effectiveness proven *in vitro*; the selection of the necessary ingredients for the formulation of semisolid topical products, for the synergistically support of the extracts effects; and the formulation and characterization of three types of topical semisolid products, intended for the prevention and treatment of the mild, moderate and severe xerosis.

CURRENT STATE OF KNOWLEDGE

1. Theoretical data on healthy and xerotic skin tissue

Chapter 1 includes information on the structural and functional dynamics of the epidermis, its functions, highlighting the skin barrier function and the essential elements in maintaining stratum

corneum hydration, the peculiarities and triggering factors of xerosis, as well as its physiological changes and symptoms, and the necessary strategies in the prevention and treatment of xerosis.

2. Identification of natural sources with applicability in xerosis and of the essential formulation criteria in the prevention and treatment of symptomatology

Chapter 2 includes relevant data from the specialized literature regarding the phytocomplexes and beneficial effects imprinted on the skin level by three plant products of therapeutic interest (*Betulae cortex*, *Liquiritiae radix* and *Avenae herba*), as well as essential data regarding the therapeutic approach and the selection criteria of pharmaceutical forms with topical application, in order to prevent and treat the symptoms of xerosis.

PERSONAL CONTRIBUTIONS

3. Obtaining plant extracts and establishing the quality standards

As a first step in the development of topical products with applicability in the management of xerosis, the valorization of selected plant products was achived by obtaining dry plant extracts enriched in active principles of therapeutic interest. Thus, it was established to perform the extraction process in two stages, with 50% ethanol as extraction solvent, and for drying by liophilization method, with the advantage of long-term preservation of the structural and functional integrity of the resulting extracts. Regarding the methodology for determining the phytochemical profile, the identification and quantification of the constituent compounds was pursued by performing a physicochemical screening. Applying spectrophotometric methods, total flavones, polyphenols and phenolcarboxylic acids contents were determined (Table III.1).

Table III.1. Results of spectrophotometric assays expressed as average ± RSD [1]

Dry plant extract	**Total flavones content (g rutoside/100g dry extract)**	**Total polyphenols content (g tannic acid/100g dry extract)**	**Phenolcarboxylic acids content (g chlorogenic acid/100g dry extract)**
Betulae extractum	3.747 ± 0.3140	47.96 ± 9.7083	25.34 ± 1.6728
Liquiritiae extractum	3.44 ± 0.3037	9.31 ± 0.9913	Undetermined
Avenae extractum	1.95 ± 0.0526	40.55 ± 6.3715	Undetermined

The results of quantitative spectrophotometric analyses revealed that *Betulae extractum* (BE) is characterized by the highest concentration of active principles, among the studied extracts, and that it was the only one for which phenolcarboxylic acids could be quantified (for the other extracts, the reaction with the Arnow reagent was negative at both high and low sample

concentrations). Also important to note is that *Liquiritiae extractum* (LE) has approximately double the amount of flavones compared to *Avenae extractum* (AE), but a four times less total polyphenols. The detection of phenolcarboxylic acids, flavones and total polyphenols in BE in significantly higher concentrations than the other dry extracts may explain the potential remarkable antioxidant effect of the birch bark extract [1].

4. Chromatographic and spectrometric characterization of dry plant extracts

For the physico-chemical characterization of dry plant extracts, the determination of the constituent bioactive compounds was performed with high precision techniques – GC-MS, HPTLC, FT-ICR-MS and FT-IR. Combining the results from the chromatographic and spectrometric methods allowed the identification and/or quantification of the constituent active compounds, as well as their confirmation and validation, due to the high precision of some of the applied methods. The characterization of the dry extracts by GC-MS revealed the main classes of compounds by the derivatization method: 56 compounds for BE – triterpenes (51.96%), saccharides (30.25%), polyols (9.45%), phenolic compounds (4.16%), fatty acids (2.31%), carboxylic acids (1.85%), and amino acids; 67 compounds for LE – saccharides (83.13%), polyols (8.42%), amino acids and derivatives (3.23%), phenolic compounds (2.39%), carboxylic acids (1.90%) and fatty acids, and 57 compounds for AE, respectively – saccharides (69.82%), carboxylic acids (21.99%), fatty acids (3.57%), polyols (2.20%), amino acids (1.78%), urea and glycolic acid. Regarding the constituent fatty acids, a specific chromatographic identification method was applied, confirming the compounds found following derivatization. From a quantitative perspective, the results highlighted that AE has a higher content of linoleic and linolenic acid compared to the other two extracts (0.19 g% linoleic acid and 0.92 g% linolenic acid), these fatty acids having an important implication in xerosis. The second selected chromatographic method, HPTLC, was applied for the identification and semi-quantitative determination of flavones and polyphenols, as well as triterpenes and saponosides, classes of compounds of therapeutic interest. Thus, concerning polyphenols quantification, rutin (6.67 µg/mL) and a mixture of lipophilic polyphenols and tannins (approx. 3mg/mL, quantified based on the chlorogenic acid standard, frequently used to express polyphenol content) were quantified for BE, and the presence of caffeic and rosmarinic acid was highlighted; for LE, rutin and caffeic acid were quantified (13.34 µg/mL and 26.67 µg/mL, respectively), and the presence of a mixture of flavones with increased lipophilicity and isoflavones was also observed; and for AE, isorhamnetin-3-rutinoside (20 µg/mL) was quantified and the presence of chlorophylls was observed. Regarding the triterpenic compounds, lupeol and betulin were identified and quantified in BE (0.338 µg/mL and 0.813

μg/mL, respectively), by the HPTLC method, and the presence of other two related compounds was also observed; while for LE, 9 bands attributed to triterpenes and saponosides were identified, and also the presence of phenolic compounds with low polarity. The results obtained from the FT-IR analysis applied to the three studied extracts highlighted, with a good probability, part of the components already identified previously, due to the vibrational fingerprint bands present in the characteristic spectrum. Based on the data from previous research in the specialized literature, the birch extract spectrum highlighted the presence of triterpenes specific bands, among which those of the betulinic acid and betulin were detected, and the presence of specific flavonoid groups; for licorice root, the presence of flavonoids, polysaccharides and glycosides was highlighted, and also of glycyrrhizin, glabridin and liquiritigenin based on their specific bands; and for the oat extract, the FT-IR spectrum identified the presence of beta-glucan and saccharides.

Currently considered one of the best and most sensitive MS techniques, due to its high resolution power (> 10^6), mass accuracy (0.1 – 2 ppm) and increased sensitivity, appreciated as the most promising analytical technique for the identification of individual molecular components, the FT-ICR-MS method confirmed most of the chemical compounds of interest found by the other methods: for birch bark extract – betulinic acid, oleanolic acid, ursolic acid, betulin, erythrodiol, betulinaldehyde, betulonic acid, lupeol, lupenone, stearic acid, oleic acid, linoleic acid, betuloside, caffeic acid, kaempferol, myo-inositol; for licorice root extract – glycyrrhizin, glycyrrhetinic acid, liquiritigenin, isoliquiritigenin, glabridin, linoleic acid, palmitic acid, myo-inositol; and in the case of oat herb extract – avenacoside A, linolenic acid, oleic acid, palmitic acid, linoleic acid, tricin, vitexin, caffeic acid, ferulic acid, tryptophan, myo-inositol, citric acid, sucrose, D-mannose, D-glucopyranose, beta-glucan, kestose. The mass accuracy (ppm) recorded for the identified compounds was very good, with values between 0.01 – 1.67, confirming their presence with high certainty.

The compounds identified and quantified for the extracts obtained indicate a phytochemical complexity, an enrichment in active principles of interest in xerotic pathology, in agreement with the specialized literature.

5. Assessment of dry plant extracts toxicity by *in vitro* and *in vivo* methods

The toxicity of plant extracts phytocomplexes can be developed through several mechanisms, such as cell membrane rupture (membrane integrity being the most frequently used characteristic), enzymatic reactions, interference in protein synthesis, irreversible binding to cell receptors and affecting the formation process of cellular genetic material [2,3]. Since not all active substances/principles have the same mechanism or level of impact, the assessment of

cellular toxicity through several experimental models, *in vitro* and *in vivo*, is a necessity to detect possible differences in the results obtained under distinct culture and test conditions. Thus, toxicological screening is an important indicator, providing valuable information at the preformulation stage.

In vitro evaluation of the extracts cytotoxicity potential was performed on the standardized normal human keratinocytes cell line (HaCaT), relevant to the targeted pathology. In these studies, the cells were exposed to increasing concentrations of the test products, over a certain period of time, based on the cellular metabolic characteristics. By correlating the two cellular parameters (the decrease in cell viability and the increase in lactate dehydrogenase, LDH, release) it is possible to correctly quantify the effect of dry extracts on cell viability. The concentrations considered for the cytotoxicity and cell viability tests were in the 12.5 – 50 μg/mL range for BE and LE, and in the 25 – 100 μg/mL range for AE. The results obtained from the analyses highlighted that *Betulae extractum* and *Liquiritiae extractum* possess a greater cytotoxic potential compared to *Avenae extractum*. At the same time, a dose-dependent relationship was spoted, especially in the case of BE, for which the highest degree of cellular damage was recorded at 50 μg/mL and the lowest at 12.5 μg/mL. For LE, almost similar results were obtained at the higher concentrations of 25 and 50 μg/mL, while the lowest toxicity was reported at 12.5 μg/mL. Unlike the other extracts, in the case of AE there was no dose-dependent relationship for the three tested concentrations (25, 50 and 100 μg/mL), the cytotoxicity values being very close. However, the cytotoxic effect was significant compared to the control ($p < 0.05$) for all extracts, at all tested concentrations. The comparative analysis of the resulting values suggests a much lower cytotoxicity in the case of AE, as it induced changes at a higher concentration than the other analyzed extracts (BE and LE), which were active at lower doses.

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. Cell viability of keratinocytes was significantly affected only by BE 50 μg/mL and LE 50 μg/mL ($p < 0.05$) [4].

In vivo evaluation of extracts toxicity was performed on *Daphnia* species, frequently used in determining the toxicity of drugs, plant extracts, various bioactive phytochemicals, and nanomaterials [5], mainly due to the increased sensitivity of these species to toxic substances, been used for 40 years in ecotoxicology as a standardized test organism [5,6]. Thus, for the

three studied dry extracts, were investigated the acute toxicity on two species of *Daphnia* – *Daphnia magna* and *Daphnia pulex*, and the teratogenic potential through a test on *Daphnia magna* embryos.

The lethality curves obtained for the extracts, plotted as L% (induced lethality expressed as a percentage) versus logarithmic concentration (Log [C]), are shown in Fig. 5.1. The results obtained from toxicity tests on *Daphnia* species for the three plant extracts (BE, LE and AE) showed different responses between the two *Daphnia* species to the extracts' species-specific susceptibilities, *Daphnia pulex* displaying increased sensitivity compared to *Daphnia magna* (Table V.1.). The lethality data indicated that while AE exhibited relatively low toxicity, for LE and BE, both species showed high levels of toxicity, especially at higher concentrations, suggesting potent bioactive compounds within these extracts.

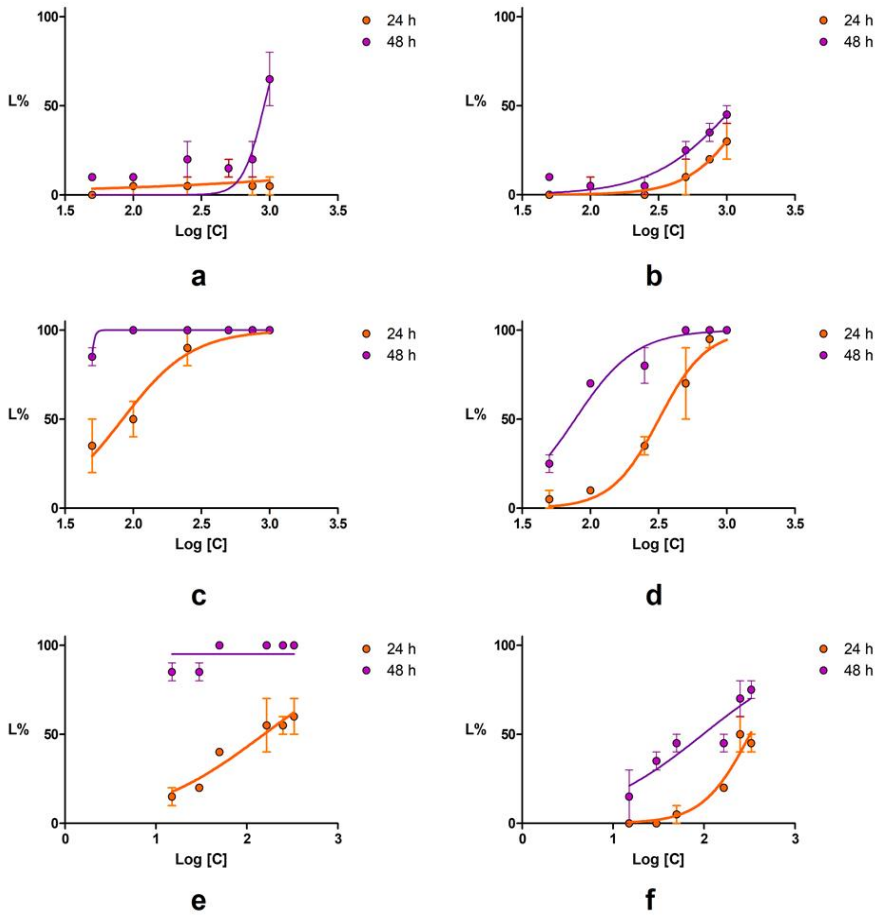

Fig. 5.1. The lethality curves obtained after 48 hours exposure of *Daphnia magna* (a, c, e) and *Daphnia pulex* (b, d, f) to the three extracts: *Avenae extractum* – a, b; *Liquiritiae extractum* – c, d; *Betulae extractum* – e, f; error bars represent the SE of two replicates (n=2)

Table V.1. Results of *Daphnia* species lethality bioassays

Specie de *Daphnia*	**Tip de extract**	**LC50 (µg/mL)**		**IC95%**	
		24 ore	**48 ore**	**24 ore**	**48 ore**

Daphnia magna	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 pulex	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%

In the embryo test assessing the teratogenic potential, the results obtained by treating *Daphnia magna* embryos with the three extracts highlighted the lack of teratogenicity at lower concentrations (15 µg/mL BE, 50 µg/mL LE and 50 µg/mL AE), as well as the complete inhibition of embryonic development in those exposed to the concentration of 500 µg/mL AE, indicating a critical toxicity threshold for this extract (Fig. 5.2.). The results of the two types of toxicity tests performed on *Daphnia* species demonstrate that the three studied plant extracts show toxic effects at high concentrations, but can be considered safe at lower concentrations.

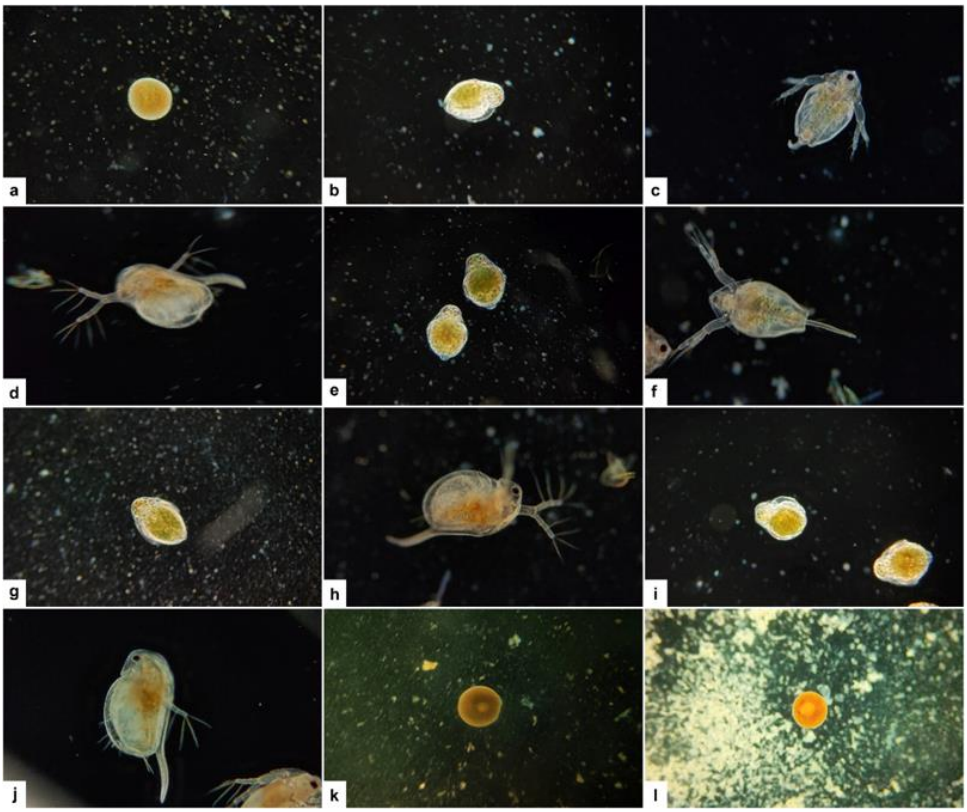

Fig. 5.2. *Daphnia magna* embryo test: a- embryos at 0 hours; b - intermediary larval stage untreated at 24 hours; c, d - young daphnid untreated at 48 hours; e - intermediary larval stage treated with 50 µg/mL LE at 24 hours; f – young daphnid treated with 50 µg/mL LE at 48 hours; g - intermediary larval stage treated with 15 µg/mL BE at 24 hours; h – young

daphnid treated with 15 µg/mL BE at 48 hours; i - intermediary larval stage treated with 50 µg/mL AE at 24 hours; j – young daphnid treated with 50 µg/mL AE at 48 hours; k, l – undeveloped embryos treated with 500 µg/mL AE at 24 and 48 hours

6. *In vitro* determination of antioxidant activity and molecular docking studies

The antioxidant potential of the studied extracts is directly correlated with the phytocomplex identified and quantified. In order to highlight the antiradical activity, the total antioxidant capacity and the potential to modulate the activity of the intrinsic systems involved in the skin natural antioxidant protection, a screening was performed by applying three methods: *in vitro* determination by chemical methods (DPPH, ABTS and FRAP) and on the standardized normal human keratinocyte cell line (HaCaT), as well as molecular docking studies (*in silico* modeling).

Following the *in vitro* evaluation of the antioxidant action by the three determination methods (DPPH, ABTS, FRAP), the resulting IC_{50} values allowed the comparison of the antiradical effect of the extracts used. Thus, it was observed that the lowest IC_{50} value was obtained, by all applied methods, in the case of birch bark extract ($IC_{50\ DPPH}$ = 0.0736 mg/mL; $IC_{50\ ABTS}$ = 0.0112 mg/mL; $EC_{50\ FRAP}$ = 0.0587 mg/mL). This activity correlates positively with the results of the determination of total polyphenols, BE having a higher content than LE and AE. For the ABTS method, the obtained results indicated different reaction kinetics for the three dry extracts, as well as the interdependence between the phytochemical profile and the free radical reaction time. BE showed a very high reaction time, much higher than other plant extracts, annihilating reactive oxygen species (ROS) in a very short time, even at very low concentrations. Also, the IC_{50} value of the birch bark extract was very close to that of the reference standard (ascorbic acid – 0.0165 mg/mL) (Table VI.1.), emphasizing a strong antioxidant action. The opposite, AE presented the weakest antioxidant activity, following the application of the ABTS and DPPH methods; however, after comparing the results obtained within the FRAP method, LE recorded the highest EC_{50} value, therefore the lowest antiradical activity. These differences that appeared between licorice root and oat herb extracts, depending on the type of antioxidant method applied, may be due to the absence of phenolcarboxylic acids that were not detected in either of these two dry extracts, which influences the ability to annihilate certain free reactive species. Also, an atypical antioxidant behavior was outlined for AE, because, although phenolic compounds could be the most important substances that significantly contribute to the antioxidant action, nevertheless the antiradical effect can also be conferred by bioactive compounds with a non-phenolic structure, substances that can contribute considerably to the total electron-donating capacity of the oat herb extract. Thus, the protective

antioxidant effect of AE may be related to the presence of fibrous non-phenolic substances and especially to the content of soluble β-glucans. The presence of these compounds justified the special antiradical profile of AE, in which greater differences were highlighted between the IC_{50} values determined by the three methods ($IC_{50\ DPPH}$ = 1.1266 mg/mL; $IC_{50A\ BTS}$ = 0.0997 mg/mL; $EC_{50\ FRAP}$ = 0.1351 mg/mL) [4].

Table VI.1. IC_{50} and EC_{50} values obtained for the three dry extracts by DPPH, ABTS and FRAP methods

Dry Plant extract	**DPPH Method, IC_{50} (µg/mL)**	**ABTS Method, IC_{50} (µg/mL)**	**FRAP Method, EC_{50} (µg/mL)**
***Betulae extractum* (BE)**	73.6	11.2	58.7
***Liquiritiae extractum* (LE)**	805.6	92.1	722.0
***Avenae extractum* (AE)**	1122.6	99.7	135.1
Vitamin C (reference substance)	16.5	-	-

By constructing a relationship map (Fig. 6.1.), it was possible to establish the links between the three antioxidant methods (ABTS, DPPH and FRAP) applied to the three groups of plant extracts (*Betulae extractum*, *Liquiritiae extractum* and *Avenae extractum*) and the concentration of bioactive compounds (polyphenols and total flavones). The result revealed a symmetry of the nodes size for all tested variables and a uniformity of the connections, which suggested the existence of a strong relationship, confirmed by using this graphical tool.

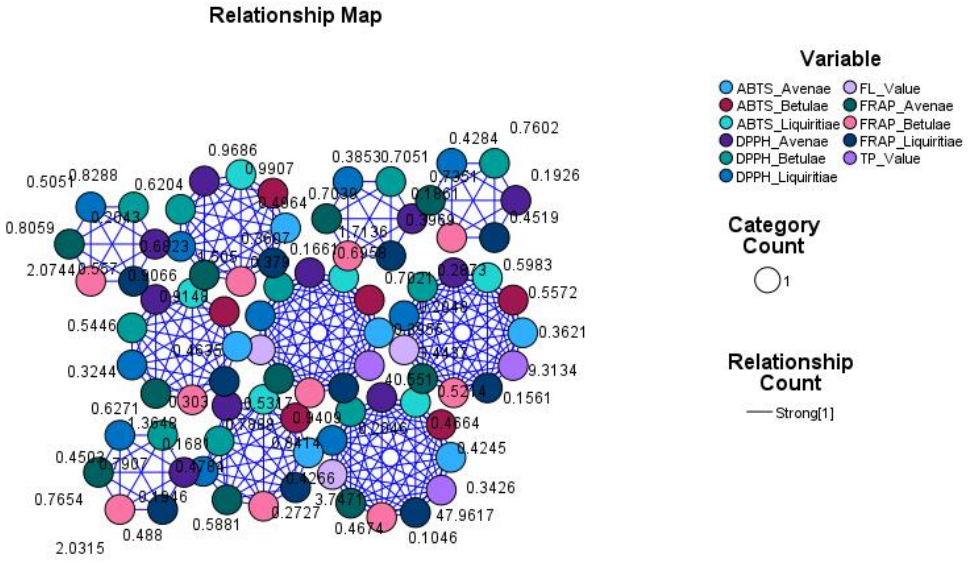

Fig. 6.1. The circle layout of the relationship map

The values of the Pearson coefficient (*r*) were negative in the case of the correlation of the antioxidant effect with the concentration of active principles, which explains the inverse correlation between the data (the higher the amount of active principles, the lower the IC_{50} value of the extracts, thus the antioxidant activity being stronger). The total polyphenol content (TPC) is directly correlated with the antioxidant activity of the plant extracts, with the compared data (TPC concentration vs. IC_{50} value) showing a very strong correlation for all antioxidant determination methods (ABTS, DPPH and FRAP) ($|r|$ is between 0.900 and 1.000). The Pearson correlation between the methodologies used in this study (DPPH, ABTS and FRAP), for the evaluation of the antioxidant activity in the extracts analyzed for each set of pairs of data obtained by different methods, showed values above 0.900, suggesting a very strong and statistically significant correlation ($p = 0.0001$) between experimentally applied methodologies. The results of the Pearson correlation between the methods ($r > 0.900$), as well as the coefficients of determination ($R^2 > 0.900$), highlighted a very well correlated antioxidant activity of the extracts showing independent values and not significantly influenced by methodological errors [4].

The antioxidant potential of the extracts was demonstrated *in vitro* and on the standardized normal human keratinocyte cell line (HaCaT), a method with greater biological relevance compared to chemical tests. After testing the selected concentrations of the extracts, based on the *in vitro* and *in vivo* cytotoxicity evaluations, it can be seen that BE possess the greatest potential at 25 μg/mL, closely followed by LE (25 μg/mL) and, respectively, of AE (50 μg/mL). Also, for the other concentrations, the antioxidant activity is very good too, higher than that of the control (ethanol). The results obtained are statistically significant ($p < 0.05$) for the 12.5 μg/mL concentration in the case of BE and LE, and for both tested concentrations (25 μg/mL and 50 μg/mL) in the case of AE.

Molecular docking experiments revealed that many of the phytocompounds identified by the screening methods have the potential to act as non-covalent ligands at the Keap1 – Nrf2 binding interface, thus the three analyzed dry plant extracts possess a high potential to exert antioxidant and anti-inflammatory effects *in vivo*, through the Nrf2 signaling pathway [4]. Several pentacyclic triterpenoids have been analyzed, such as betulin, betulinaldehyde, betulinic acid, betulonic acid, lupeol, lupenone, oleanolic acid and ursolic acid. Among these compounds, ursolic acid showed the best binding potential towards the Kelch domain of the Keap1 protein. Glycyrrhizin and its aglycone, glycyrrhetinic acid, showed the highest binding affinity for the Kelch domain of Keap1, forming the largest number of polar interactions with residues in the binding site. Also, cinnamic acid showed very good binding affinity and formed

five electrostatic interactions. In the case of liquiritigenin, the results showed a potential binding conformation in the Kelch domain of Keap1, with the possibility of disrupting the Keap1–Nrf2 interaction and promoting the nuclear translocation of Nrf2. Through molecular docking simulations, it was observed that isoliquiritigenin also forms many favorable non-covalent interactions with the binding pocket, as well as that chlorogenic acid binds to Cys151 in a similar manner with satisfactory binding energy. Docking studies also yielded interesting results for kaempferol (flavonol), vitexin (apigenin-glucoside-type flavone) and tricin (O-methylated flavone).

7. *In vitro* assessment of dry extracts impact on cell migration rate as part of the re-epithelialization process

The *in vitro* cell migration test ("scratch test") represents a valuable method in evaluating the skin restructuring process in preclinical studies, contributing to a better understanding of the mechanisms by which the regulation of the cell migration process takes place, as well as to obtaining preliminary results regarding the effects produced on migration under different experimental conditions, such as exposure to different active substances, plant extracts, etc. [13,14]. The experimental model applied to the standardized normal human keratinocyte cell line (HaCaT) comprised two series of cells: 1). a series of unstimulated cells maintained in culture, in the presence of the tested extracts, for 24 hours; 2). a series of cells stimulated inflammatory and oxidative by the addition of 15 ng/mL tumor necrosis factor (TNF-α) and 0.1 μM Phorbol myristate acetate (PMA), mimicking non-specific inflammation and stress oxidative, maintained in culture in the presence of the tested extracts for 24 hours. Important parameters in wound healing were tracked – wound width was calculated (average width of the cell-free zone per time unit - μm), further used in the estimation of wound confluence (percentage of the initial wound area covered by migrating cells over time - %) and of the maximum wound healing rate - $\mu m^2/h$.

The results obtained in the *in vitro* cell migration studies support and confirm the effectiveness of these plant extracts, especially the birch bark extract (Fig. 7.1.) in the re-epithelialization process, also highlighting a potential anti-inflammatory effect, by increasing the rate of cell migration in conditions of induced non-specific inflammation, associated with oxidative stress. Among the concentrations tested for the three selected extracts, with a potential contribution to the stimulation of cell migration, and implicitly, to the development of the re-epithelializing effect, by (1) maintaining the homeostasis of the epidermal extracellular matrix, (2) restoring the extracellular matrix, (3) significantly increasing the rate of wound healing, the

following stood out: *Betulae extractum* 3 µg/mL, *Liquiritiae extractum* 7.5 µg/mL and *Avenae extractum* 7.5 µg/mL [4].

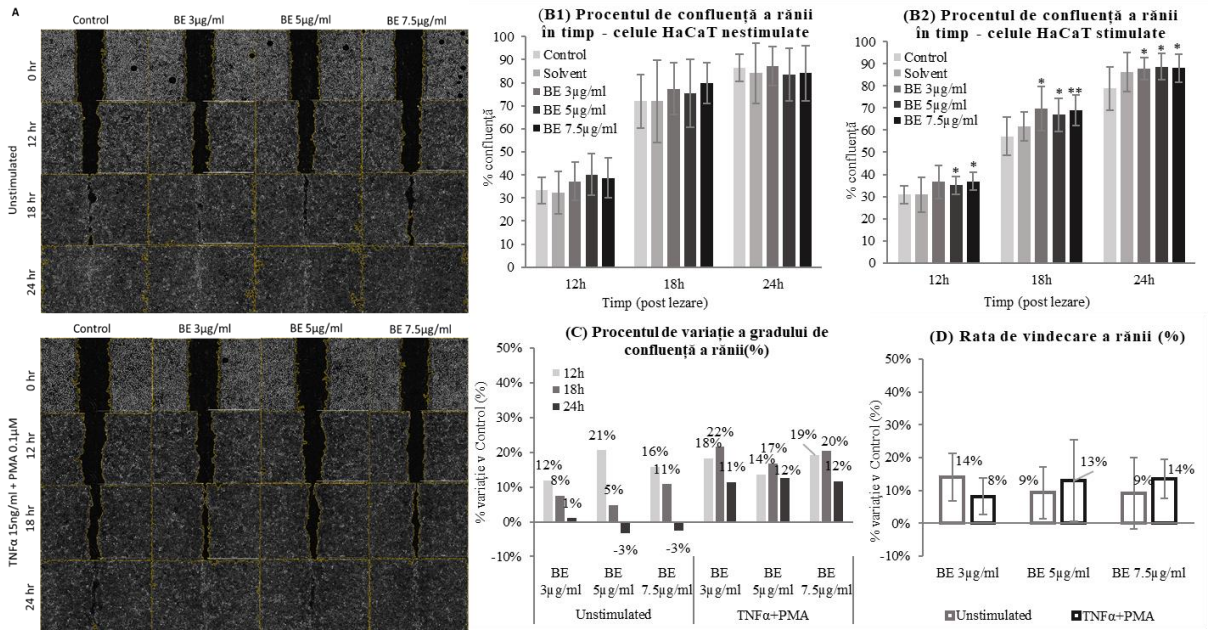

Fig. 7.1. Representative images from the *in vitro* wound healing assay, illustrating cell migration into the wound site (marked area); image acquisition was performed for 24 h using the 4x magnification objective in bright-field high-contrast mode (scale bar = 1000 µm). Evaluation of wound healing in the presence of dry birch bark extract using image-based cellular analysis: the evolution of the degree of wound confluence after 12h, 18h, and 24h, respectively, for cells treated unstimulated (B1) and stimulated pro-inflammatory with 15 ng/mL TNF-α and 0.1 µM PMA (B2); Calculation of percent variation for degree of wound confluence (C) and wound healing rate (D). All numerical values are represented as mean (n=3) ± standard deviation (SD); (*$p < 0.05$; **$p < 0.01$)

8. Statistical analysis of correlations between active principles content – *in vitro* and *in vivo* cytotoxicity – *in vitro* effectiveness

In order to obtain a deeper understanding of the connections between the results obtained at the determination of the active principles content by spectrophotometric methods with those resulting from the *in vitro* and *in vivo* assessment of cytotoxicity and with the quantified effects by the *in vitro* tests of cell migration and determination of antioxidant activity, statistical correlation analyses were applied. The Principal Component Analysis (PCA) highlighted the correlations between variables, while the heatmaps evidenced the differences between all three studied extracts, regarding biaoctive compounds and pharmacological properties. All significant statistical results obtained with the correlations between bioactive constituents and pharmacological potential of all three extracts (hierarchical clusters and heatmap analysis)

validate the possible clinical relevance of determinations performed. 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.

9. Formulation of semisolid topical products using the dry extracts

In order to synergistically support the effectiveness of the studied dry extracts, a series of active ingredients (glycerin, niacinamide, urea, ceramides, vitamin E, dimethicone, beeswax, squalene from olive oil) and functional ingredients (acryloyldimethyl-ammonium laurate copolymer/VP, xanthan gum, emulsifiers, triglycerides, ethylhexylglycerin) were selected, based also on their skin compatibility. Starting from the symptomatology described for each stage of xerosis and from the formulation approaches necessary to treat them, three types of formulations were selected: gel for skin dryness tendency (mild xerosis), cream-gel for a reduced degree of skin dryness (moderate xerosis) and cream for a moderate degree of skin dryness (severe xerosis). Taking into account the phytocomplex identified and quantified, their effectiveness demonstrated *in vitro*, the cytotoxicity results from *in vitro* and *in vivo* assessments, as well as the recommended manner of administration, the concentrations and the therapeutic direction were established: the dry extract obtained from oat herb – moisturizing gel, for the tendency to dry skin, in a concentration of 5% solution 0.3 mg/mL; the dry extract obtained from licorice root – moisturizing and emollient cream-gel, for reduced skin dryness, in a concentration of 3% solution 0.5 mg/mL; the dry extract obtained from birch bark – moisturizing, emollient and repairing cream, for moderate degree of skin dryness, in a concentration of 2% solution 0.3 mg/mL. Birch bark extract can also be applied in very severe xerosis, as an adjunct to topical anti-inflammatory therapy.

The characterization of the developed semisolid topical formulations highlighted the obtaining of stable, homogeneous products with appropriate organoleptic properties, in accordance with the quality requirements indicated in the specialized literature. From the rheological behavior perspective, the products have a low viscosity, ensuring a very good spreading capacity, thus allowing an easy application, without additional aggression to the skin tissue - an important characteristic for products intended for the prevention and treatment of xerosis. Combining the studied dry extracts with carefully selected active ingredients, customized according to the physiological changes characteristic of the targeted xerosis stage, allowed the development of complex topical products, capable of repairing the existing deficits at the epidermis level, as well as stimulating the intrinsic mechanisms of restoring skin homeostasis.

FINAL CONCLUSIONS AND PERSONAL CONTRIBUTIONS

General Conclusions

The research performed as part of the Ph.D. thesis aimed to identify some plant products with potential in the prevention and treatment of xerosis, the physico-chemical and pharmaco-toxicological characterization of the obtained extracts, as well as the development of three topical products intended for the personalized treatment of xerosis, depending on the severity of the symptoms.

- Three plant products were selected and included in the study – *Betulae cortex* (birch bark), *Liquiritiae radix* (licorice root) and *Avenae herba* (aerial parts harvested before flowering), all presenting in the specialized literature data related to the beneficial effects in the treatment of dermatological conditions, including xerosis, with the exception of oat herb, for which less evidence was found about the therapeutical potential in dermatological field.
- In order to obtain plant extracts enriched in active principles, especially polyphenols, triterpenes, fatty acids, saccharides (compounds of interest in xerosis), 50% ethanol was chosen as the extraction solvent, allowing the extraction of both hydrophilic and lipophilic compounds.
- For the dry plant extracts, obtained by optimezed processes of concentration in a rotary evaporator and drying by lyophilization, a phytochemical screening was performed by applying spectrophotometric, chromatographic and spectrometric analyses, for the qualitative and quantitative determination of phytocomplexes. Since each analysis method has detection limitations, it's more useful to apply a set of techniques, in order to confirm and validate the presence of the constituent compounds.
- The quantitative spectrophotometric determinations for flavones, total polyphenols and phenolcarboxylic acids, revealed a higher concentration for the birch bark extract, thus justifying its higher therapeutic potency, compared to the other two dry plant extracts. Regarding the content of phenolcarboxylic acids, it was quantified only for the birch bark extract (for the other extracts, the reaction with the Arnow reagent was negative, both at high and at low concentrations of the sample).
- The gas chromatography – mass spectrometry (GC-MS) analysis highlighted the diversity of the phytocomplexes obtained, through the derivatization method identifying a total of 56 compounds in the case of the birch bark extract (mainly triterpenes, saccharides, phenolic compounds, fatty acids), 67 compounds in the case of licorice root extract (mainly saccharides, amino acids, phenolic acids, fatty acids) and respectively 57 compounds in the case of oat herb extract (mainly saccharides, carboxylic acids, fatty acids, amino acids, urea). Concerning the

previously identified fatty acids in the three plant extracts by the derivatization method, they were also confirmed by a GC-MS specific identification method. From a quantitative perspective, the oat extract is characterized by a richer content, both in linoleic acid and in linolenic acid, compounds of therapeutic interest in xerosis.

- The high-performance thin layer chromatography (HPTLC) allowed the identification and semi-quantitative determination of polyphenols and triterpenes from the three studied dry extracts: for birch extract – quantification of rutin and of a lipophilic polyphenols and tannins mixture (intense blue-colored band, with Rf = 0.991), and identification of cafeic and rosmarinic acids; for licorice extract – quantification of rutin and cafeic acid, and identification of a lipophilic flavones and isoflavones mixture (intense orange-colored band, with Rf > 0.99); for oat extract - isorhamnetin-3-rutinoside quantification and chlorophylls identification (intense red-colored band). Regarding triterpenes, lupeol and betulin were identified and quantified in the birch bark extract by HPTLC method, and also the presence of other two compounds related to betulin or lupeol were also observed; for the licorice extract, a number of saponosides and triterpenes were identified, and also some phenolic compounds with low polarity.
- The results obtained with by the FT-ICR-MS analysis (high-resolution Ion-Cyclotron-Resonance Fourier Transform mass spectrometry) confirmed the presence of a large number of active principles in the composition of the three studied dry extracts, validating the results obtained by GC-MS analysis: for *Betulae extractum* – triterpenes (betulin, betulinic acid, lupeol, lupenone, oleanolic acid, ursolic acid, erythrodiol), fatty acids (stearic acid, oleic acid, linoleic acid), polyphenols (caffeic acid, kaempferol) and myo-inositol; for *Liquiritiae extractum* – triterpenes (glycyrrhizin, glycyrrhetinic acid), polyphenols (liquiritigenin, isoliquiritigenin, glabridin), fatty acids (palmitic acid, linoleic acid); for *Avenae extractum* – avenacoside A, fatty acids (linoleic acid, linolenic acid, oleic acid, palmitic acid), flavones (tricine, vitexin), polyphenols (caffeic acid, ferulic acid), amino acids (tryptophan), saccharides.
- FT-IR analysis revealed some of the components identified by GC-MS and FT-ICR-MS techniques, due to the vibrational fingerprint bands present in the characteristic spectrum: the birch extract spectrum highlighted the presence of triterpenes specific bands, among which those of the betulinic acid and betulin were detected, and the presence of specific flavonoid groups; for licorice root, the presence of flavonoids, polysaccharides and glycosides was highlighted, and also of glycyrrhizin, glabridin and liquiritigenin based on their specific bands; and for the oat extract, the FT-IR spectrum identified the presence of beta-glucan and saccharides.

- The assessment of the cytotoxicity of the dry plant extracts was performed *in vitro*, on standardized normal human keratinocytes cell line (HaCaT), relevant for xerotic pathology, and *in vivo*, on *Daphnia magna* and *Daphnia pulex* models. LDH and MTS assays, performed on HaCaT, demonstrated that at higher concentrations (range 25 – 100 μg/mL) birch and licorice extracts showed more pronounced cellular toxic effects, while oat extract showed more reduced. However, at lower concentrations (< 12.5 μg/mL for birch and licorice extracts, and < 50 μg/mL for oat extract), the results revealed a minimal negative impact on keratinocytes, plant extracts being considered safe. Regarding the *in vivo* toxicity assessment, the two types of toxicity tests on *Daphnia* species (acute toxicity and teratogenic potential) have demonstrated the same results as those obtained on HaCaT – that the three studied plant extracts show toxic effects at high concentrations, but can be considered safe at lower concentrations low (12.5 μg/mL for dry birch and licorice extracts, 50 μg/mL for dry oat extract).
- The antioxidant activity of the three dry plant extracts was assessed by *in vitro* chemical methods (DPPH, ABTS and FRAP) and on a standardized normal human keratinocyte cell line (HaCaT), as well as by *in silico* studies. The association of these three types of determinations allowed highlighting the antiradical activity, the total antioxidant capacity, as well as the ability to modulate the activity of the intrinsic systems involved in the skin natural antioxidant protection of the three studied extracts – by the Nrf2 signaling pathway. The obtained results demonstrated good antioxidant effects, in direct correlation with the identified phytocomplexes, the highest antioxidant capacity being attributed to the birch bark extract and the weakest to the oat herb extract.
- The results obtained in the *in vitro* cell migration studies, performed on standardized normal human keratinocytes cell line (HaCaT), support and confirm the effectiveness of these plant extracts in the re-epithelialization process, also highlighting a potential anti-inflammatory effect, by increasing the rate of cell migration in conditions of induced non-specific inflammation (tumor necrosis factor, TNF-α) and oxidative stress (Phorbol myristate acetate, PMA).
- Taking into account the phytocomplex identified and quantified, their effectiveness demonstrated *in vitro*, the cytotoxicity results from *in vitro* and *in vivo* assessments, the therapeutic direction of the dry extracts studied was established: the dry extract obtained from oat herb – tendency to skin dryness (mild xerosis); the dry extract obtained from licorice root – reduced degree of skin dryness (moderate xerosis); dry extract obtained from birch bark - moderate degree of skin dryness (severe xerosis). *Betulae extractum* it can also be applied in very severe xerosis, as an adjunct to topical anti-inflammatory therapy.

- Starting from the symptomatology described for each stage of xerosis and from the formulation approaches necessary to treat them, three types of topical formulations were selected – gel for mild xerosis (tendency to dryness), cream-gel for moderate xerosis (reduced degree of skin dryness) and cream for severe xerosis (moderate degree of skin dryness).
- The characterization of the developed semisolid topical products highlighted the obtaining of stable, homogeneous formulations with appropriate organoleptic properties, with a very good spreading capacity, thus allowing the easy application, without additional aggression to the skin tissue - an important feature for products intended for the prevention and treatment of xerosis.
- The combination of studied dry extracts with carefully selected active ingredients, customized according to the physiological changes characteristic of the targeted xerosis stage, allowed the development of complex formulas, capable of repairing the existing deficits at the epidermis level, as well as stimulating the intrinsic mechanisms of restoring skin homeostasis.

Degree of Originality

The original elements of the thesis are: highlighting the potential of the extract obtained from the aerial parts of oats, harvested before flowering, in xerotic pathology, based on the composition enriched in saccharides, amino acids and fatty acids, useful in correcting skin imbalances, but also based on the good antioxidant activity, important in restoration of the skin barrier; *in vitro* evidence of the ability to stimulate cell migration (on keratinocytes) of the three dry extracts, with an important impact on the re-epithelialization process; highlighting the anti-inflammatory potential of the dry extracts, by increasing the rate of cell migration in conditions of induced non-specific inflammation (through stimulation with TNF-α), associated with oxidative stress (through stimulation with PMA); the customized therapeutic approach of the xerosis stages, through the development of three topical products personalized in terms of composition, in close connection with the mechanisms necessary to treat the degree of damage and, consequently, the symptomatology.

Research Perspectives

In the furure, we propose to performe stability studies, in accelerated and long-term conditions, for the three dry extracts and the three developed topical products, *in vitro* release studies, skin compatibility studies, as well as clinical effectiveness studies, on healthy volunteers, with the application of some techniques for testing the topical product impact on SC hydration (corneometry), SC integrity (TEWL), elastic properties of the skin (cutometry), erythema (mexametry), determination of skin pH values (pH-metry) and the influence on the skin microrelief – roughness, scaling (visioscan).

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