Assessment of the biologically active potential of some apitherapeutic products with addition of natural oils in experimental skin wounds in rats

(SUMMARY OF Ph.D. THESIS)

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INTRODUCTION

Bee products are natural products that currently are enjoying increased attention among consumers, as well as in the global pharmaceutical industry. Honedew honey is one of the main products of the hive and it is highly valued due to a wide range of compounds that make it up, improving its nutritional profile and healing potential. Among the many benefits, researchers mention the anti-inflammatory, antimicrobial, antiseptic, antioxidant properties of this type of honey. All these activities are essential in various repair processes, in which apitherapeutic products find their applicability, including in tissue regeneration process. Propolis, another valuable product of hive is considered by many specialists, a basic ally in the therapy of various diseases, including skin pathologies. Another category of natural products with high potential for clinical use in the field of dermatology is represented by natural oils. Globally, interest in natural oils has considerably grown in recent decades, due to the wide range of applications, which are directly related to a number of pharmacological and biological activities.

Despite all the evidence gathered over time on the positive health effects of natural products, particularly bee products and natural oils, there is still slight reluctance to use them in medical clinics. Therefore, it is the duty of researchers to make sustained efforts to conduct as many studies as possible in order to rediscover and capitalize on natural remedies with many therapeutic uses long forgotten or sometimes considered empirical.

PURPOSE AND OBJECTIVES OF THE THESIS

The main purpose of the present doctoral thesis consisted of the elaboration of two apitherapeutic products with addition of natural oils and their implementation as topical therapy in experimental skin wounds in Wistar rats. In order to achieve the major goal, the following general objectives have been set:

- Evaluation of the quality physico-chemical parameters of honeydew honey sample, used as common basis for the two apitherapeutic products studied and verification of its compliance with the legislative framework;
- Compositional analysis of sea buckthorn oil, respectively of thyme essential oil, used in formulation of the apitherapeutic products subjected to testing, in correlation with highlighting the main bioactive compounds of great importance in skin repair processes;
- Research on the content of biologically active compounds of the investigated apitherapeutic formulas, compared to honeydew honey, their common basis;
- Evaluation of total antioxidant capacity of the formulated apitherapeutic

products, in correlation with the antioxidant potential of honeydew honey;
- ❖ Comparative study on antibacterial activity of the apitherapeutic products and components used;
- ❖ Testing the regenerative effect of the developed apitherapeutic products on experimentally induced skin wounds in laboratory rats.

THESIS STRUCTURE

The doctoral thesis entitled "Assessment of the biologically active potential of some apitherapeutic products with addition of natural oils in experimental skin wounds in rats" is structured in two usual parts, comprising 152 pages, 17 tables and 64 figures.

PART I : Current stage of knowledge

The Ist part is intended for the current state of knowledge and it groups the first three chapters, each one presenting general information summarized from literature and in accordance with the objectives pursued in this research.

Chapter I is entitled "Correlative aspects regarding skin functions and skin regenerative mechanisms" and it includes 3 subchapters, which address notions related to skin structure and functions, mechanisms involved in skin wound healing and *in vivo* experimental models commonly used to monitor the evolution of regenerative tissue process.

Chapter II, entitled "General considerations regarding bioactive testing of bee products and natural oils", it is divided in two subchapters, which present the bioactive properties of natural products, such as: the antioxidant potential of bee honey and propolis, respectively the antibacterial properties of bee products and natural sea buckthorn and thyme oils.

Chapter III is entitled "Current data regarding skin wounds therapy with some bee products and natural oils" and it includes 2 subchapters, which complete the whole picture of the first part, through data on use of honey and propolis in the treatment of skin wounds and the benefits of sea buckthorn and thyme oils in wounds.

PART II : Personal contribution

The IInd part consists of personal contribution, which is structured in six chapters, intended to address various issues formulated in the pursued objectives.

Chapter IV is called "Purpose and objectives of the research" and it consists of setting out six general objectives in order to achieve the main purpose.

Chapter V is entitled "Peculiarities in which the research was carried out" and it summarizes the experimental products and animals introduced in the testing, as well as all specialized laboratories, both within UASVM (University of Agricultural Sciences and

Veterinary Medicine) Cluj-Napoca and outside of it, which served to conduct the experiments.

Chapter VI refers to the "Materials and methods" used during the research, namely: biological material, physico-chemical analyses performed, specific determinations performed, organization and description of the experimental protocol established, and methods of statistical analysis used. The implemented experimental protocol was based on: surgical procedures, monitoring the evolution of wound healing process by macroscopic and histological examination, bacteriological examination of the microbism present on animals skin, before and after surgery.

Chapter VII groups the "Results and Discussions" part, including six subchapters in which, the data obtained for each objective are outlined.

Chapter VIII summarizes the part of "Conclusions and recommendations" resulting from the investigations that were carried out.

Chapter IX is called "Originality and innovative contributions of the thesis" and it analyzes the innovative part and contribution of this study to all existing data in the literature.

RESEARCH RESULTS

The investigations conducted in this doctoral thesis were carrried during 2018-2020. In this context, the strategy of approaching the research topic included two main directions: the analysis of compositional quality and the evaluation of biological properties of two apitherapeutic products, which were implemented as topical therapy in experimental skin wounds in rats.

The experimental products introduced in this study were two apitherapeutic products, developed in collaboration with SC VITAPLANT SRL, Săcel, Sibiu county, Romania. The apitherapeutic product I was obtained by mixing two natural components, represented by honeydew honey (95%) and sea buckthorn oil (5%) per 100 g. The apitherapeutic product II was obtained by mixing two bee products: honeydew honey (89%), respectively soft propolis extract (10%), and thyme essential oil (1%), per 100 g. The raw material necessary in the elaboration of the two apitherapeutic products was provided by SC VITAPLANT SRL.

Fig. 1. The investigated apitherapeutic products (original)

The tested animals were represented by 15 rats (*Rattus norvegicus*), Wistar line, clinically healthy, males, one year old, with an average body weight of 518.47±14.43 g. During the experiment, they were maintained within the UASVM Cluj-Napoca Biobase, under specially created conditions and in compliance with the current legislation (ISO STANDARD 10993-2/2004; DIRECTIVE 2010/63/EU OF EUROPEAN PARLIAMENT AND EUROPEAN COUNCIL; LAW 43/2014 FROM ROMANIA). In this paper, we have also performed a series of usual physico-chemical analyses within specialized laboratories and according to standardized methods established in literature.

Study of physico-chemical parameters of honeydew honey

In general, physico-chemical properties of honey are considered true parameters of its quality, their investigation being necessary in order to assess the authenticity of this product and to identify the factors responsible for its depreciation. The obtained results revealed the conformity of the studied honey sample with the legal provisions and its classification as high quality honeydew honey.

All physico-chemical parameters analyzed indicated values situated within the reference limits provided by the legislative framework, including: water content (16%); electrical conductivity (940 μS/cm^{-1}); free acidity (10.20 meq NaOH/kg); pH (3.84); HMF (0.27 mg/kg honey); sugars profile (glucose+fructose content = 71.33%), diastase activity (37.12 DN); total lipids (0.024%); total proteins (0.55%). We also noticed the absence of tetracycline residues and the melissopalynological analysis highlighted the presence of specific honeydew elements.

Study of compositional quality of natural oils

The analysis of data obtained for the investigated sea buckthorn oil revealed a wide range of biologically active compounds, which activate the physiological functions of skin and stimulate the process of tissue regeneration and collagen synthesis, such as: total carotenoids (142.33 mg /100 g oil); saturated fatty acids (41.30%), including palmitic acid (41.03%), stearic acid and myristic acid; monounsaturated fatty acids (53.50%), including palmitoleic acid as the major compound (35.12%), but also oleic and vaccenic acids; polyunsaturated fatty acids (5.20%), including linoleic acid (3.63%) and linolenic acid; tocopherols represented by α-tocopherol (128 mg/100 mL oil), β-tocopherol (6.24 mg/100 mL oil) and *δ*-tocopherol (0.65 mg/100 mL oil); sterols, represented by stigmasterol (32 mg/100 mL oil) and β-sitosterol (3000 mg/100 mL oil).

The analysis of data obtained when testing thyme essential oil revealed 21 different organic compounds. Of these, the major compound was thymol, which recorded high values (52.15%) and it is known for its strong antibacterial activity. We also reported important amounts of *p*-cymene (23.55%) and γ-terpene (5.31%). The values obtained confirmed that the investigated sample belonged to thymol chemotype, and the other minor compounds presented values ranging from 0.03%-3.81%.

Study of the content of biologically active compounds from honeydew honey and apitherapeutic products

The evaluation of total polyphenol content revealed average values of 87 mg GAE/100 g for honeydew honey, 973 mg GAE/100 g for apitherapeutic product I and 997 mg GAE/100 g for apitherapeutic product II. Consequently, we can observe an increase in total polyphenol content in case of the two apitherapeutic products compared to honeydew honey sample, which served as their common basis. These aspects can be attributed to the additional constituents that are part of the products, such as sea buckthorn oil for apitherapeutic product I, respectively thyme essential oil and soft propolis extract for apitherapeutic product II.

The evaluation of flavones-flavonols content revealed the following values: 33.33 mg QE/100 g for the apitherapeutic product I, respectively 37.33 mg QE/100 g for the apitherapeutic product II. In contrast, the amount of flavones-flavonols contained in honeydew honey sample was lower (20.33 mg QE/100 g honey), proving once again, the superiority of the apitherapeutic products, due to the additional constituents from their composition.

Study of total antioxidant activity of honeydew honey and apitherapeutic products

The evaluation of antiradical activity by DPPH method revealed various values, depending on the content of antioxidant compounds present in each sample. The lowest percentage of inhibition was observed in honeydew honey (21.03%), while in apitherapeutic products, we obtained much higher values. The apitherapeutic product II showed the highest percentage of inhibition (84.29%), while the apitherapeutic product I obtained a lower value (41.75%).

The evaluation of antioxidant activity by FRAP method also revealed different values. The highest FRAP value was reported in apitherapeutic product II (2734 μM Fe^{2+}/100 g product), followed by apitherapeutic product I (541.3 μM Fe^{2+}/100 g product), honeydew honey showing the lowest antioxidant activity (29.68 μM Fe^{2+}/100 g honey).

Study of antibacterial activity of the apitherapeutic products and components used

The results regarding antibacterial activity of the two apitherapeutic products, as well as of each component of their formula are expressed according to diameters of the inhibition zones (mm).

The bacterial strains tested were classified as: "sensitive", if the diameter was greater than 12 mm, "moderately sensitive" when the diameter was between 6 and 12 mm and "resistant", if the diameter was less than 6 mm. The six international reference

bacterial strains used in this study showed different sensitivity to the investigated apitherapeutic products (Table 1).

The clinically isolated staphylococcal strains showed different sensitivity to the action of the apitherapeutic products, the most sensitive proving to be the *Staphylococcus spp. 354* strain. In general, Gram-positive bacteria were more sensitive to the action of the tested products, but good results were also obtained regarding the Gram-negative ones.

Table 1

The antibacterial potential of the investigated samples

Nr./ No.	TULPINA BACTERIANĂ/ BACTERIAL STRAINS	PROBELE TESTATE/ TESTED SAMPLES						
		Ulei de cimbru/ Thyme oil	Ulei I de cătină/ Sea buckthorn oil	Miere de mană/ Honeydew honey	Produs apiterapeutic I/ Apitherapeutic product I	Produs apiterapeutic II/Apitherapeutic product II	Extract moale de propolis / Soft propolis extract	Amoxicilină/ Amoxycilin
		DIAMETRUL ZONEI DE LIZĂ (mm)/ DIAMETER OF THE LYSIS AREA (mm)						
1.	*S. aureus 6538 P*	18.09	0	8.71	10.13	14.22	9.63	23.47
2.	*Staphylococcus spp. 88*	18.54	0	11.47	14.32	14.64	7.89	28.58
3.	*Staphylococcus spp. 112*	24.18	0	8.32	8.59	12.04	9.46 IP	11.64 CR
4.	*Staphylococcus spp. 119*	24.19	0	8.04	9.13	13.07	10.52	26.50
5.	*Staphylococcus spp. 183*	23.89	0	8.44	9.47	10.85	9.92	9.53
6.	*Staphylococcus spp. 354*	39.02	0	12.15	15.63	17.08	14.01	40.86
7.	*B. cereus. 11778*	26.33	0	9.06	10.37	18.73	13.40	R
8.	*L. monocytogenes 13932*	23.20	0	13.66	14.58	16.80	15.13	30.12
9.	*E. coli 10536*	27.06	0	0	8.77	9.83	0	15.39
10.	*S. enteritidis 13076*	17.62	0	8.93	9.78	11.05	0	18.23
11.	*Ps. aeruginosa 27853*	21.19 IP	0	15.93 CR	15.93 CR	17.49	0	R
12.	*S. pseudintermedius*	27.68	0	9.32	12.36	13.74	9.77	24.38

Legend: IP = partial inhibition; CR = resistant colonies; R = resistant.

Study of healing and regenerative potential of the apitherapeutic products

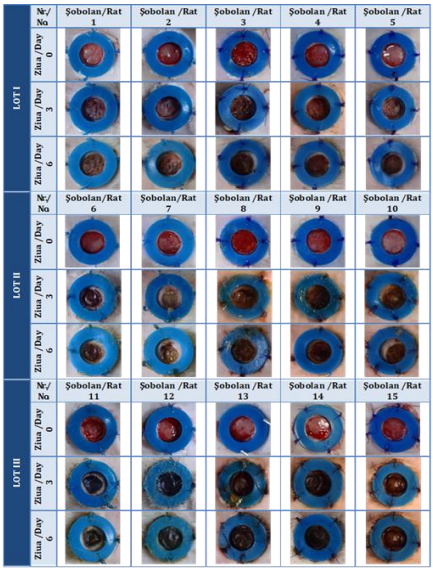
Following the investigations carried out on the basis of the implemented experimental protocol, focused on the induction of excisional wounds by using an 8 mm biopsy punch in rats divided into three groups (batch I-untreated wounds; batch II-wounds treated with the apitherapeutic product I; batch III-wounds treated with apitherapeutic product II), we noticed different aspects:

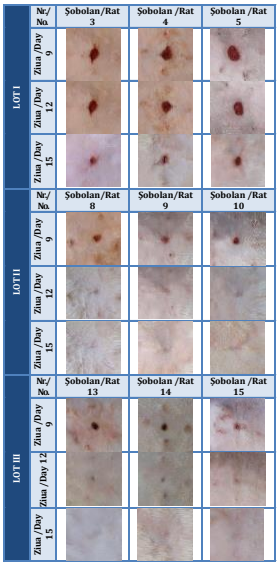
- Both control and treated wounds were covered with crusts, but there were also some differences. Thus, in most control skin wounds, the crusts were sero-hemorrhagic, reddish and strongly adherent to the underlying tissue. In contrast, the treated wounds were much drier, more voluminous and brown in subjects belonging to group II and brownish-black in those belonging to group III (Table 2);
- The wounds of the subjects treated with product I, respectively with product II revealed a complete healing on the 12th day, but with easily visible scar residues. In comparison, the untreated wounds still had significant size and concave aspect on the 12th day, post-intervention (Table 3);
- The healing-regeneration process was entirely completed on the 15th day of the experiment in subjects treated topically with the apitherapeutic products (groups II and III), hardly distinguishing the place of skin defect, while in the control group, the wounds were still present (Table 3).

Table 2 and 3

The evolution of wound healing process on days

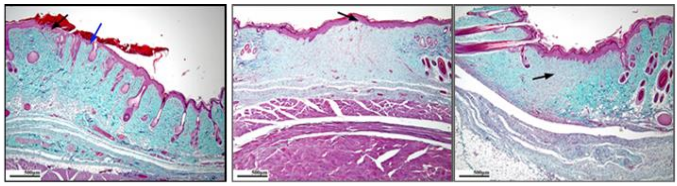
A (days 0-3-6) ***B (days 9-12-15)***





- Starting with the 9th day post-intervention, the wound closure percentage (%), which was calculated for each rat, revealed an average value of 93.77% for group II and of 95% for group III, while in group I, the average value was 77.93%;

- On the 12th day of the experiment, the closure percentage was 100% for the wounds treated with the apitherapeutic products from groups II and III, with discrete scar marks, and for the control group, it reached the average value of 80.90%. At 15 days after the experiment, a wound closure percentage of 93.11% was recorded in the untreated group;
- The comparative microbiological analysis of pre- and post-intervention samples collected from rats skin revealed a lower number of colonies developed on the 7th post-therapeutic day in the experimental groups, compared to the control group. This very small number of colonies compared to day 0, we attributed it to the antibacterial effect of the experimental products;
- The histological evaluation of skin biopsies showed a good therapeutic effect regarding topical application of the two apitherapeutic products, skin repair processes being much more advanced at the end of the experiment; the treated groups presented a thick epithelium, that was almost completely restored (Fig. 2).



A B C

Fig. 2. Histological aspect of cutaneous epithelium in rats from the investigated batches: A-Batch I: black arrow-area without epithelium; blue arrow-epithelium in the process of consolidation; B-Batch II: black arrow-recovered epithelium above the intervention area; C-Batch III: black arrow-restored area, without hair and glands (Col. Trichrome Goldner; original).

CONCLUSIONS AND RECOMMENDATIONS

- ✓ The physico-chemical parameters analysis of the honeydew honey sample, used as a common basis for the two tested apitherapeutic products, revealed its compositional quality and its compliance with the current legislative framework.
- ✓ The compositional analysis of sea buckthorn and thyme oils, components of the apitherapeutic formulas, indicated a rich content of bioactive compounds with special impact in tissue repair processes.
- ✓ The obtained results also highlighted a rich content of total polyphenols, respectively of flavones-flavonols of the investigated apitherapeutic products.
- ✓ The results of our study revealed good antibacterial and antioxidant activities of the tested apitherapeutic products, with beneficial effects in wound healing.
- ✓ The implementation of excisional model in testing the tissue regenerative potential of two apitherapeutic products with addition of natural oils proved to be effective in the investigations performed, the repair tissue processes being dominant throughout the experiment.
- ✓ Starting from the rich arsenal of biotherapeutic products currently existing on the market, we recommend their development and wider use in the field of veterinary medicine.
- ✓ Carrying out and reproducing future studies with different types of local honey and natural oils regarding their biologically active potential.
- ✓ Based on the bioactive properties reported in this paper, we recommend the implementation of this type of apitherapeutic formulas in veterinary clinics, as a topical therapy in the management of simple and complicated wounds in animals.

REFERENCES

1. BERTONCELJ J., DOBERŠEK U., JAMNIK M., GOLOB T., 2007. Evaluation of the phenolic content, antioxidant activity and colour of Slovenian honey, *Food Chem. 105, 822–828*.
2. BETANCES-SALCEDO E., REVILLA I., VIVAR-QUINTANA A.M., GONZÁLEZ-MARTÍN M.I., 2017. Flavonoid and Antioxidant Capacity of Propolis Prediction Using Near Infrared Spectroscopy, *Sensors (Basel) 17(7):1647.*
3. BIALEK A., BIALEK M., JELINSKA M., TOKARZ A., 2016. Fatty acid profile of new promising unconventional plant oils for cosmetic use, *Int. J. Cosmet. Sci. 38, 382–388*.
4. BITTENCOURT M. L. F., RIBEIRO P. R., FRANCO R. L. P., HILHORST H. W. M., DE CASTRO R. D., FERNANDEZ L. G., 2015. Metabolite profiling, antioxidant and antibacterial activities of Brazilian propolis: *Use of correlation and multivariate analyses to identify potential bioactive compounds, 76, Part 3:449–457.*
5. BLASER G., SANTOS K., BODE U., VETTER H., SIMON A., 2007. Effect of medical honey on wounds colonised or infected with MRSA, *J. Wound Care 16(8), 325–328.*
6. BOATENG J., DIUNASE K.N., 2015. Comparing the antibacterial and functional properties of cameroonian and manuka honeys for potential wound healing-have we come full cycle in dealing with antibiotic resistance, *Molecules 20:16068–16084*.
7. BOBIŞ OTILIA, MARGHITAS LIVIU, KRISZTINA RINDT, NICULAE MIHAELA, DEZMIREAN DANIEL, 2008. Honeydew honey: correlations between chemical composition, antioxidant capacity and antibacterial effect mierea de mană: corelatii între compoziţia chimică, capacitatea antioxidantă şi efectul antibacterian, *Lucrări ştiinţifice Zootehnie şi Biotehnologii, Timisoara 41.*
8. CHUA L.S., LEE J.Y., CHAN G.F., 2013. Honey protein extraction and determination by mass spectrometry, *Analytical and Bioanalytical Chemistry 405(10), 3063–3074.*
9. COOPER R., 2016. Honey for wound care in the 21st century, *Journal of Wound Care 25(9): 544–552, doi:10.12968/jowc.2016.25.9.544.*
10. CORNELL K., 2012. Wound Healing. In: Tobias, KM.; Johnston, SA., editors, *Veterinary Surgery: Small Animal 1, Vol. 1, St. Louis: Saunders, 125-134.*
11. COSTA M.F., DURÇO A.O., RABELO T.K., BARRETO R.S.S., GUIMARÃES A.G., 2019. Effects of Carvacrol, Thymol and essential oils containing such monoterpenes on wound healing: a systematic review, *J Pharm Pharmacol. 71(2):141-155.*
12. COTRAN R.S., ABBAS A.K., FAUSTO N., ROBBINS S.L., KUMAR V., 2005. ROBBINS & COTRAN: Patologia - Bases Patológicas das Doenças, 7. ed. *Rio de Janeiro*: *Elsevier, 1592 p.*
13. COTTE J.F., CASABIANCA H., CHARDON S., LHERITIER J., GRENIER-LOUSTALOT M.F., 2004. Chromatographic analysis of sugars applied to the characterisation of monofloral honey, *Anal. Bioanal. Chem. 380, 698–705.*
14. COUQUET YVES, ALEXIS DESMOULIÈRE, MARIE-LAURE RIGAL, 2013. Les propriétés antibactériennes et cicatrisantes du miel, *Actualités Pharmaceutiques, Volume 52, Issue 531, Pages 22-25, ISSN 0515-3700*