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Formulation and investigation of topical dosage forms containing natural active substances

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1. Introduction and aims

Nowadays, there is a growing demand for natural remedies and herbs. More and more natural food supplements and medicines are appearing on the pharmaceutical market, not to mention that 80 percent of the population in developing countries uses traditional medicine and herbs in various indications. The use of topical preparations containing active ingredients of natural origin for preventive or therapeutic purposes avoids the undesirable effects caused by synthetic ingredients. However, before the application of herbs and other active substances of natural origin pre-formulation and various analytical tests must be carried out in order to the determination of qualitative and quantitative determination of the compounds contained in them. During the formulation, the most common problem is the poor water solubility of the active ingredient and that their ability to penetrate biological membranes is low due to the high molecular weight of the active compounds. These facts may reduce the bioavailability of the component and thus the extent of the therapeutic effect.

The biggest challenge in formulation of topical dosage forms is to increase the diffusion capacity of the active ingredients and to achieve penetration, as it is very difficult to pass through the top layer of the skin, the stratum corneum (SC). There are many methods to overcome skin barrier properties, including physical and chemical techniques as well. The most common and appropriate method is to use special excipients, called Chemical Enhancers (CEs), which are able to transport the molecules with them to the SC or modify its structure, thus allowing the drug to enter. Therefore, the use of various excipients, including surfactants, is key in the formulation of ointments, creams and gels, which may facilitate the diffusion of the active ingredient through the skin and thus increase the therapeutic effect. The main object of my research was the selection of the appropriate excipients for the formulation of external pharmaceutical preparations, with special regard to the use of surfactants to increase solubility and penetration.

As the topic of my doctoral dissertation, I chose two natural active ingredients with different therapeutic effects, *Spirulina platensis* (*S. platensis*) and *Calendula officinalis* (*C. officinalis*) flower for the formulation of external medicinal products.

The *Spirulina platensis* algae is one of the most important representatives of cyanobacteria and has been used in medicine both internally and externally for centuries. Its therapeutic effect is due to the wide range of bioactive components it contains. The pigment called phycocyanin, which gives a blue-green color and a significant antioxidant effect, is found in the highest amount in this algal species. In addition, it contains an outstanding

amount of one of the essential antioxidant enzymes, the metal-containing superoxide dismutase (SOD), and the content of beta-carotene, vitamin E and gamma-linolenic acid is also really high.

Cardozo et al. described that unsaturated fatty acids, essential amino acids, vitamins, minerals and antioxidants extracted from *S. platensis* promote wound healing and inhibit the development of inflammatory processes, and its secondary metabolites have antibacterial and antiviral activity. **Nihal** and his research team have demonstrated that phycocyanin-rich ointment can be used successfully in the topical treatment of acne vulgaris, as it also has bactericidal activity against *Cutibacterium acnes* (*C. acnes*) and *Staphylococcus epidermidis*.

The yellow flower and leaves of *C. officinalis* are used as an alternative or complementary therapy in the form of infusions, scalds, tinctures or other extracts, most often in creams and ointments. In India, ointments contain *C. officinalis* flowers are used to treat wounds, scars, and herpes, while the leaves are used in the form of scabs for the local therapy of varicose veins. The carotenoids in it have anti-inflammatory properties, while the flavonoids have antioxidant and antimicrobial activity. Its main flavonoid component is quercetin, which exerts its significant antioxidant activity by neutralizing reactive oxygen and nitrogen radicals, but also targets inflammatory signaling pathways such as STAT1, NFκB, and MAPK. Marigold extract has a significant anti-inflammatory effect by inhibiting the synthesis of proinflammatory cytokines, the enzyme COX-2 and prostaglandin. **Javadi et al.** investigated the anti-inflammatory and analgesic effects of orally administered quercetin in their clinical study in patients with rheumatoid arthritis. They found that quercetin significantly reduced joint pain, thereby improving patients' quality of life. Limited information is available in the literature on the effects of the external application of quercetin on arthritis. However, **Bodhankar et al.** described that calendula oil improves the *in vitro* percutaneous absorption of diclofenac sodium when applied in a Carbopol gel.

As a dosage form, creams were designed to treat acne vulgaris using *S. platensis*, while with the usage of *C. officinalis* extract the aim was to formulate an ointment, cream or gel for the adjunctive treatment of rheumatoid arthritis with the addition of diclofenac sodium and methyl salicylate.

The primary goal was to select the appropriate ointment, cream and gel ingredients as well as penetration enhancers to achieve the desired level of bioavailability. With the help of different types of emulsifiers, we wanted to formulate dosage forms for topical use with favorable drug release. *Spirulina platensis* powder (SPP) with different types of nonionic surfactants (Tefose 63 (TFS), sucrose ester SP 70 (SP70), Polysorbate 60 (P60), and

Cremophor A6:A25 (CR)) was used in our preparations. To enhance the permeability of SPP and to improve its solubility, Transcutol HP was also added to the formulations. In the case of the *C. officinalis* different types of surfactants (sucrose ester SP 70 and Empicol LZ/N) and Carbomer type gel-forming agents (Synthalen K, Carbopol 974P and Pemulen TR-1) were used for the formulation of creams and gels.

One of our objectives was to compare the compositions in terms of the amount of drug dissolved using a Franz vertical diffusion cell and a spectrophotometer. Free radical scavenging activity of the formulations was measured to demonstrate the beneficial antioxidant effect of the formulations. Investigation of possible adverse effects was also considered important, so MTT cytotoxicity measurements were performed on the HaCaT cell line.

The antibacterial activity of *S. platensis*-containing creams was tested on *C. acnes* and *S. aureus* bacterial strains which are involved in the pathogenesis of acne vulgaris, while with the formulation of *C. officinalis*, diclofenac sodium, and methyl salicylate containing ointment, creams and gels the goal was to evaluate its therapeutic efficacy in rheumatoid arthritis.

2. Materials and methods

2.1. Materials

The Transcutol HP and Tefose 63 were a gift from Gattefoss, while the sugar ester SP70 was a generous gift from Sisterna. The Cremophor A6 and A25 were provided by BASF. Cetyl stearyl alcohol, stearic acid, propylene glycol, isopropyl myristate and Aknemycin ™ ointment were purchased from Hungaropharma Ltd.

Empicol LZ/N emulsifier for the formulation of marigold creams was purchased from Innospec Performance Chemicals Italy SRL, while Synthalen K was provided by Elton Corporation SA. Diclofenac sodium, methyl salicylate and triethanolamine were obtained from Merck KgaA. White Vaseline and lanolin were products of Zhongbao Chemicals Co., Ltd.

The non-essential amino acid solution required for cell culture, the penicillin-streptomycin mixture, the GlutaMax ™ supplement, the 96-well plates, and the cell culture flasks were provided by Thermo-Fisher. The HaCaT cell line (human keratinocyte cells) was ordered from the Cell Lines Service.

The *Spirulina platensis* used in the studies was obtained from the culture collection of the Autotrophic Organizations (CCALA) of the Institute of Botany by Prof. Dr. Gábor Vasas, Head of the Department of Botany. The collection of *Calendula officinalis* (*C. officinalis*) flowers took place in Romania from June to August 2019 by the staff of the University of Oradea.

All other excipients and reagents are from Sigma-Aldrich (Budapest, Hungary).

2.2. Preparation of extracts

S. platensis was cultured in Zarrouk's medium in batch culture at pH 8.2 and 20 ± 1 ° C with a light / dark light period of 12 hours. Algal filaments were collected by centrifugation after 12 days of culture and then lyophilized at the Department of Botany.

A mixture of ethyl alcohol and distilled water was used as a solvent to extract the bioactive compounds of *C. officinalis* from the plant. One hundred grams of *C. officinalis* flower samples were placed in a flask and 1000 ml of a 96% ethanolic solution was added. The mixture was extracted twice in an ultrasonic bath for 90 minutes at 45 ° C and then filtered through a cellulose membrane (0.45 μm pore diameter). The residue was extracted several times with 60% ethanol and water (1:1) under the same conditions. After extraction,

the alcoholic fraction was removed by centrifugation and the supernatant was evaporated to dryness on a rotary evaporator and lyophilized.

2.3. Formulation of external dosage forms

Algae-containing creams were formulated using various nonionic surfactants, Polysorbate 60 (P60), Cremophor A6:A25 (1:1) (CR), Tefose 63 (TFS), and sugar ester SP 70 (SP70). The amount of each surfactant in the compositions was 3-3%, while the active ingredient (*S. platensis*) was 5-5%. In my experimental work, I formulated creams in which *S. platensis* was present in a suspended form, as well as formulations in which the algae was dispersed in a form dissolved in Transcutol in order to the better drug release.

In the case of *C. officinalis*, one ointment, two creams and three gels were formulated. The active ingredients were diclofenac sodium, *C. officinalis* flower extract and methyl salicylate in the same amounts in both composition, but they differed significantly in the type of the vehicle.

2.4. Dosage form tests

The pH of the formulations was determined immediately after formulation and after 30 and 60 days (stored at 21 ° C throughout) to detect any stability problems.

Texture analysis was performed to estimate the mechanical properties of the formulations the day after formulation. A compression tests were carried out in which the resistance of the formulations was quantified using a Brookfield CT3 Texture Analyzer and associated software.

Rheological measurements were performed to characterize the physicochemical properties of the formulated ointment, creams and gels. Flow and viscosity properties were determined with a RheolabQC rotary rheometer. Data were analyzed using RheoPlus Rheometer software. The flow and viscosity curves of the formulations were determined by rotational tests with controlled shear rate using shear stress and viscosity.

The *in vitro* release of the active ingredients and the diffusion tests were carried out using a Franz diffusion cell. The receptor chambers of each diffusion cell were filled with 30% ethanol. Synthetic cellulose acetate membranes were impregnated with isopropyl myristate (IPM) for 30 minutes before use. A sample of 1.00 g was applied to each membrane and they were placed on top of the receptor phase.

The test was performed at a temperature of 32 ± 1 ° C by shaking the receptor medium continuously (350 rpm) with a magnetic stirrer.

The phycocyanin content of the samples containing *Spirulina platensis* was measured at 620 nm with a UV-VIS spectrophotometer using 30% ethanol as a blank. For ointment, creams and gels containing *C. officinalis*, the diclofenac sodium content of the receptor medium was detected at 275 nm and the methyl salicylate at 237 nm. The main component of the *C. officinalis* extract, quercetin, was measured at 370 nm.

2.5. The methodology of cell culturing

Sterile DMEM (Dulbecco's modified Eagle's medium) medium was used as cell culture medium. HaCaT cells were grown in a plastic cell culture flask under a humidified CO_2 atmosphere (5% v/v) at 37 ° C. Cell culture medium was changed twice a week under a laminar flow sterile cabinet. When the confluent cell layer formed, the cells were passaged under aseptic conditions, and then 1 million cells were placed in a new sterile flask and supplemented with medium to 10 ml. Cytotoxicity and antioxidant experiments were performed on HaCaT cells with a minimum of 20 and a maximum of 40 passages.

2.6. Cytotoxicity study

The in vitro cytotoxicity assay was performed on a HaCaT cell line. Cells were seeded in a 96-well cell culture plate (104 cells / well) and allowed to grow in a CO_2 incubator at 37 ºC for 7 days until a confluent cell layer was formed.

During the assay, the cell culture medium was removed from the cells, and the samples, PBS and Triton-X 100 (10 v/v%) solutions were pipetted onto the cells (200 µl/well) and incubated for 30 minutes at 37 °C. Then the solutions were aspirated and cells were washed twice with 1 ml PBS. I incubated the cells with MTT dye for an additional 3 h. The solution was then removed from the cells and the resulting purple formazan crystals were dissolved in isopropanol (1.0 N hydrochloric acid = 25: 1). Finally, absorbance was measured at 570 nm with a background of 690 nm. The values obtained correlated with the number of living cells. Cell viability was expressed as a percentage of the negative control.

2.7. Investigation of antioxidant effect

To evaluate the antioxidant activity of each preparation, the amount of superoxide dismutase enzyme was determined and 2,2-diphenyl-1-picrylhydrazil (DPPH) assay were performed.

In the first experiment, I exposed HaCaT cells to UVB radiation for 10 min before or after treatment with the given compositions, thus inducing oxidative stress. During pretreatment, the medium was removed from the cells, 200 µl of test solution or control sample was added, and the cells were incubated with the samples for an additional 20 min. After 20 min of UV-B irradiation and removal of samples, the cells were washed twice with PBS. For the post-treated group, cells were irradiated for 20 min before treatment, incubated with test solutions for 20 min, and then washed with PBS. The cells were then harvested and centrifuged, and the SOD activity of the supernatant was determined using a Cayman kit according to the manufacturer's instructions by colorimetric measurement.

In the second assay, 1.0 ml of 0.06 mM DPPH radical solution was pipetted into a 2.0 ml sample, and the reaction mixtures were incubated for 30 minutes in the dark. When DPPH reacts with antioxidant compounds, these compounds transfer hydrogen to DPPH. When DPPH donated the hydrogen radical, the reaction resulted in a color change from dark violet to light yellow. Absorbance was measured with a UV spectrophotometer at 517 nm using absolute ethanol as a background. The absorbance values obtained can be used to determine the degree of antioxidant activity (% inhibited reactive oxygen species (ROS)).

2.8. Investigation of the antibacterial activity of formulations containing *Spirulina platensis*

The antibacterial effect of the preparation containing *S. platensis* was studied on the bacterial strains of *Staphylococcus aureus* (*S. aureus*) and *Cutibacterium acnes* (*C. acnes*) by a standard microdilution method in collaboration with the staff of the Institute of Medical Microbiology of the University of Debrecen.

During the experiment, 100 µl of bacterial inoculum was pipetted into a 96-well plate, and then 100 µl of test solution was added. The plates were incubated for 6 hours at 37 ° C in an aerobic environment and in a Concept 400 anaerobic chamber. During the incubation, samples were taken at 2 and 6 hours and their absorbance was measured at 600 nm.

2.9. Investigation of the anti-inflammatory effect of formulations containing *Calendula officinalis*

To evaluate the therapeutic effect of *C. officinalis*, we performed a randomized prospective study in 115 patients with abarticular rheumatism treated at the Bihile Băile Felix Rehabilitation Clinical Hospital in Romania. The active group was treated with the formulation containing C. officinalis, diclofenac sodium and methyl salicylate, while the placebo group with gel which contained no active ingredients twice daily for 14 days. Treatments were performed with formulations formulated with Synthalen K gelling agent (with / without active ingredients).

All patients in the study completed a pain assessment questionnaire, the Visual Analog Scale (VAS), on which patients could place their pain intensity anywhere on a 10 cm long line, "no pain" (level 0) and "unbearable pain" (level 10) with verbal descriptors at the endpoints.

In the clinical study, investigation of the knee or shoulder was performed with both longitudinal and transverse high-frequency ultrasound to measure the thickness of the entire synovial membrane.

3. New scientific results

3.1. Results of the measurements conducted with the *Spirulina platensis* containing creams

3.1.1. Macroscopic properties and pH

The evaluation of the macroscopic properties of the formulated creams was performed immediately after preparation and after 30 and 60 days, respectively. Formulations containing the active ingredient in a dissolved form had a homogeneous, pale bluish-green appearance, while the suspension type compositions were heterogeneous. The pH of each formulation ranged from 6.5 to 7.0 and did not change significantly after 60 days, indicating the stability of the creams.

3.1.2. Texture analysis

According to the results of compression tests, different compositions required different compression forces, so they had different consistencies. Comparing the formulations containing *S. platensis* in suspended and dissolved form, there were significant differences. As expected, the formulations containing the lyophilized drug dissolved in Transcutol showed less strain against deformation than the suspension variants. I compared the non-active creams with the formulations containing *S. platensis* prepared with the same excipients. According to the study, the application of *S. platensis* did not change statistically the value of the compression force in any of the cases.

3.1.3. *In vitro* release and diffusion test

The experiments predicted that formulations containing *Spirulina platensis* in dissolved form had higher diffusion values than their suspension pairs containing the same emulsifier. The results show that the formulations containing Tefose 63 (TFS) or sugar ester SP70 (SP70) had a better diffusion profile than Cremophor (CR) and Polysorbate 60 (P60) emulsifiers, even without the use of Transcutol. The maximum diffused amount of phycocyanin was 39.81 ± 0.83% (9.18 ± 0.38 mg) for TFS and 39.80 ± 1.15% (9.18 ± 0.41 mg) by examining the composition formulated with the sugar ester.

3.1.4. *In vitro* cytotoxicity study on HaCaT cell line

The cytotoxicity of 0.50% (w/w) solutions of nonionic surfactants, Transcutol and algae, and each preparation were investigated on a HaCaT cell monolayer.

Based on the results, it can be said that the type of emulsifier greatly affected the viability of the cells. The most significant toxicity was detected for P60 and CR emulsifiers, where cell viability was less than 50% compared to the negative control group (phosphate buffered saline (PBS)). Cells treated with SP70 sugar ester had the highest viability (93.05 ± 2.20%), followed by TFS treatment with 82.79 ± 2.95 percent. Thus, both SP70 and TFS emulsifiers showed minimal toxic effects on keratinocyte cells at concentrations of 0.50% (w/w).

Examination of the formulations demonstrated that the creams without Transcutol caused higher cell viability values than the formulations containing the same emulsifier and Transcutol as well.

3.1.5. *In vitro* antioxidant studies of preparations containing *Spirulina platensis*

For the *in vitro* antioxidant experiment, formulations containing TFS and SP70 as emulsifiers were selected based on the results of diffusion studies and MTT cytotoxicity tests. I used two types of assays to evaluate the antioxidant activity.

In the first experiment, in the group exposed to ultraviolet radiation, where I did not apply pre- or post-treatment with the test solutions, the level of superoxide dismutase (SOD) enzyme was significantly reduced compared to the group which was not treated with UV. As a consequence of the pretreatment, a smaller decrease, i.e. higher enzyme activity, was observed in terms of SOD. The best results were obtained by premedication with the sugar ester and Transcutol containing formulation (containing *S. platensis* in dissolved form), in which case the average value of SOD activity after UVB irradiation was 26.45 ± 0.85%. However, based on my results, it can be said that the post-treatment could not prevent the significant decrease in enzyme levels caused by UVB radiation.

Based on the values obtained in the DPPH test, the antioxidant activity of the formulations containing the active ingredient was found to be significantly higher than that of the formulations of the same composition but without the active ingredient. According to our results, the formulations containing the active substance in a dissolved state were able to significantly reduce the free radical DPPH, i.e. they had significant radical scavenging

activity. The formulation containing SP70 sugar ester and Transcutol also showed the most effective antioxidant activity according to the results of the DPPH assay.

3.1.6. Investigation of the antibacterial effect

To test the effect of *S. platensis* creams against acne vulgaris, the most favorable formulations containing TFS and SP70 emulsifiers with Transcutol were selected based on the assays described above.

Based on the results of the standard microdilution assay, both formulations showed higher antibacterial activity against *C. acnes* after two hours of incubation, compared to *S. aureus*. After six hours, however, the effectiveness of the formulations against *S. aureus* increased. Overall, our results showed that *C. acnes* was more sensitive to treatments.

3.2. Results of the measurements conducted with the *Calendula officinalis* containing formulations

3.2.1. Macroscopic properties and pH of preparations containing *Calendula officinalis*

The ointment and the creams prepared had a homogeneous orange appearance, while the gels were clear and these properties did not change after 60 days. Our products have met the official requirements for visual, olfactory and tactile characteristics. Based on the results of the pH measurement, it can be concluded that a suitable pH has been achieved in terms of formulation and therapeutic effect. The stability of the formulations is indicated by the fact that the pH values did not decrease or increase significantly after 60 days.

3.2.2. Texture analysis

According to the measurements, and as might be expected, different compositions required different amounts of compressive force. The resistance of the gels showed a significantly lower value compared to the ointment and cream formulations. The results of the compression test show that gels are more suitable formulations due to the easier application. The highest force was measured for the composition containing Vaseline and lanolite (140.33 ± 5.13 N for the composition containing the active ingredient and 146.00 ± 7.54 N for the composition without the active ingredient). This formulation with a less soft consistency may

inhibit the release of the active ingredients and may impair patient compliance due to more difficult application.

3.2.3. Rheological characterization

The rheological analysis provided information on the flow properties of the formulated semi-solid formulations as well as the degree of thixotropy by determining the rheograms and viscosity. As the results of the study show, the characteristic properties of the ointment, cream and gel base used (hydrocarbon gel, o/w emulsion ointments or hydrogel) may affect the rheological behavior of the formulations.

Significant thixotropic and pseudoplastic behaviors were observed for all formulations except vaseline and lanolin. Each formulation was structurally viscous, i.e., a shear thinner material, as the viscosity decreased with increasing shear rate in all cases, and the formulations became more fluid. As expected, lower viscosity values were observed for the gels than for the ointment and creams. The lowest viscosity value was detected for the gel formulated with Synthalen K gelling agent.

3.2.4. *In vitro* release and diffusion studies

The in vitro diffusion profile of diclofenac sodium, methyl salicylate and quercetin through an isopropyl myristate (IPM) impregnated cellulose acetate membrane was determined. Comparing the drug diffusion profiles from ointment, creams and gels, it can be concluded that all three drugs were present in higher amounts in the receptor phase for gels. According to the study, the formulation containing Synthalen K gel-forming polymer proved to be the most suitable. In this case, the cumulative amount of diclofenac sodium after 2 hours was 79.62 ± 0.91% (7.96 ± 0.09 mg) and that of methyl salicylate was 53.37 ± 1.86%, while the amount of quercetin was 45.01 ± 0.91% (12.04 ± 2.24 mg).

3.2.5. The effect of *Calendula officinalis* on cell viability

The results showed no significant difference in the effect of formulations containing drugs and formulated without active ingredients on cell viability. The vaseline and lanolin-based formulation showed high cell viability (88.92 ± 5.97%), while treatment with Empicol LZ/N emulsifier resulted in the greatest decrease in cell viability. In this case, viability was only 68.43 ± 5.62%, probably due to the presence of the anionic emulsifier. Of the

formulations, the gel formulated with Synthalen K proved to be the least toxic, with a viability of 93.61 ± 1.49% for cells treated with this formulation and was therefore well tolerated.

3.2.6. Antioxidant effect of medicinal products containing *Calendula officinalis*

According to the DPPH test, the free radical scavenging activity of *C. officinalis* extract was 65.34 ± 2.10%. Significant differences were observed between the formulations containing and without active ingredients in each case. Overall, the antioxidant activity of the formulations containing *C. officinalis* extract was significantly higher than that of the same formulations, but without the extract. Among the preparations, only the formulation containing Synthalen K achieved 50% free radical neutralizing activity, so it is considered to be the most effective composition. The lowest activity was measured for the vaseline and lanolin formulation, which proved to be an unfavorable ointment base for drug release.

3.2.7. The evaluation of the therapeutic effect – a human clinical trial

The anti-inflammatory and analgesic effects of the selected Synthalen K gel formulation were based on a randomized, placebo-controlled study. Our results showed a significant reduction in pain between the first and 14th day of treatment based on the visual analog scale (VAS). In patients whose knees were affected by rheumatic disease, the mean baseline was 6.06 ± 1.21 in the active group and 6.18 in the placebo group. On day 14 of treatment, the value on the VAS scale decreased to 2.36 ± 0.89 in the study group and to 5.48 ± 0.54 in the placebo group.

The results obtained in the ultrasound study showed a significant reduction in synovial membrane thickness in patients treated for knee arthritis in the active group (from 3.22 ± 1.14 to 1.32 ± 0.91), while in patients with rheumatoid arthritis involving the shoulder, the initial thickness decreased from 3.41 ± 1.39 to 1.92 ± 1.07 by the end of day 14.

4. Discussion

In the case of preparations containing active ingredients of natural origin, particular attention should be paid to the design of the appropriate dosage form, with special attention to the selection of excipients.

In our first experiment, the consistency, diffusion profile and biocompatibility of formulations containing lyophilized *Spirulina platensis* algae with different surfactants were investigated and compared, and then the antibacterial activity of the selected formulations was evaluated. During the cream formulation, in addition to the compositions containing *S. platensis* algae in suspension form, creams containing the powder in a dissolved state were also formulated with the help of Transcutol. For the preparation of creams, nonionic surfactants, Polysorbate 60, a mixture of Cremophor A6 and A25, sugar ester SP70 and Tefose 63 were chosen for their gentler effect on the skin.

Considering the results of the texture analysis and the *in vitro* diffusion study, it was found that formulations containing Transcutol in combination with SP70 sugar ester or Tefose 63 emulsifier as a penetration enhancer had a softer consistency and therefore a better release and diffusion profile across the cellulose acetate synthetic membrane. Transcutol has been shown to be an excellent solubilizer, incorporating the active ingredient in dissolved form into creams, which may have affected the penetration and bioavailability of the active ingredients. It also has the advantage of being non-toxic and biocompatible with the skin according to the literature.

In vitro cytotoxicity studies are essential to determine the safety profile of medicinal products. The effect of the different compositions on cell viability was investigated by performing an MTT test on a HaCaT cell line. According to the literature, HaCaT cell line is proposed as an *in vitro* model to study the function of keratinocytes and to investigate the biocompatibility of preparations for use on the skin surface. Based on the results of the study, the effect of SP70 and TFS surfactant formulations on cell viability was more favorable than that of P60 and CR surfactant formulations.

Topical antibiotic therapy is usually the first choice in the treatment of acne vulgaris. However, increasing bacterial resistance leads to the decreasing efficacy of the topical antibiotics as monotherapy and requires combination treatment with other topical agents. In acne vulgaris, the bacteria *C. acnes* and *S. aureus* are mainly responsible for dermatitis, which can induce local inflammation by producing chemotactic factors, and attracted neutrophils release inflammatory mediators such as ROS in the dermis. Neutralization of ROS can

significantly reduce the cellular damage associated with inflammation during acne, so in addition to antimicrobial activity, preparations with antioxidant activity may be more effective in the treatment of acne vulgaris. In our assays to determine antioxidant activity, HaCaT cells were exposed to UV radiation before or after treatment with *S. platensis*-containing creams, and SOD activity was determined. SOD, one of the major antioxidant enzymes found in large amounts in *S. platensis*, neutralizes free radicals generated during oxidative stress, thus preventing cell damage. Our measurements showed that the drug dissolved in Transcutol in combination with the surfactant TFS and SP70 resulted in increased SOD activity compared to the suspension formulations. Our results indicated that the efficacy of the pretreatment was significantly higher for the formulations containing dissolved *S. platensis*, while we did not find any difference between the efficacy of the pretreatment and post treatment for the suspension formulations.

By measuring the radical scavenging capacity of DPPH, we have demonstrated that the incorporation of the active ingredient in solution into a given formulation can increase its permeability and thus its effectiveness.

In the antibacterial test, our preparations were also tested on *S. aureus* and *C. acnes* bacteria. The exact mechanism of the action of *S. platensis* is unknown, but some hypothesized that its antimicrobial activity may be related to the synergistic effect of its fatty acid components and polysaccharide content. According to our studies, the formulated creams reduced the viability of *C. acnes* and *S. aureus* compared to the negative control groups. Compositions containing the SP70 sugar ester surfactant reduced bacterial viability to a greater extent after 6 h of incubation than those prepared with the TFS surfactant. These results also showed that the type of surfactant affected the bioavailability and antimicrobial activity of the drug.

In the second part of the experiments, I formulated six different topical formulations with an anti-inflammatory effect containing the combination of diclofenac sodium, methyl salicylate and *C. officinalis* flower extract. Unlike the first series of experiments, different types of ointment and cream bases were developed for this study, such as a hydrocarbon gel containing vaseline and lanolin, and creams with a higher water content in an o/w emulsion system that emulsified by anionic (Empicol LZ/N) or nonionic (SP70 sugar ester) emulsifiers. Macromolecular hydrogels were also formulated with various excipients (Carbopol 974P, Pemulen TR-1 and Synthalen K), from which more favorable drug release and diffusion were expected.

Several studies have already described the inverse relationship between the viscosity of semi-solid dosage forms and the diffusion capacity of the active ingredients in them. According to our experiments, the Synthalen K gel formulation showed the best diffusion results and the lowest viscosity, the softest consistency. The higher diffusion rate of the gels in our *in vitro* diffusion assay is thus due to their low viscosity, which resulted in increased drug release. In addition, gels are easier to apply, making them more suitable for treating large surfaces.

According to the MTT test, Synthalen K was the least toxic of the various formulations, with a cell viability of 93.61 ± 1.49% and this composition had the highest antioxidant activity. The composition containing vaseline and lanolin as the vehicle showed the high cell viability (88.92 ± 5.97%), while treatment with o/w emulsion-type ointments resulted in a greater decrease in viability.

The results of the preformulation studies and the antioxidant test suggested the composition containing the gelling agent Sythalen K to be suitable for *in vivo* testing. Our product was subjected to a prospective, placebo-controlled clinical trial to evaluate its anti-inflammatory and analgesic effects. Pain is a symptom whose intensity is felt most by the patient concerned, so he or she is able to accurately assess himself or herself. Therefore, a VAS scale was used to assess pain intensity. The method is simple, non-invasive and easy to use, allowing the patient to classify pain. No clinical data are currently available on the clinical trials of the concomitant use of diclofenac sodium, methyl salicylate and *C. officinalis* extract, however, several clinical studies of diclofenac gels in patients with rheumatoid arthritis have been reported. In a randomized clinical trial described in the literature, diclofenac gel was shown to be superior to placebo in the treatment of pain over a 3-day treatment period, as expected. The gel we formulated showed a significant reduction in VAS-assessed pain after a 14-day treatment period. Several studies have reported that the short-term synovial response to topical anti-inflammatory therapy, i.e., a decrease in synovial thickness, can be detected by ultrasound, so ultrasound imaging was performed before and after treatments. The results showed that after treatment with the gel formulation prepared with Synthalen K, a significant reduction in synovial thickness was observed compared to the negative control group, so that the treatment had a beneficial effect on local inflammation.

Our experimental work has pointed out the differences between ointment, cream and gel formulations with different excipients, surfactants, and gelling polymers. The excipients affected the rheological behavior, the cytotoxicity and the release of the active substances and the degree of antioxidant effect.

5. Conclusion

During my PhD studies, I formulated external dosage forms in various therapeutic indications using herbal active ingredients. The primary goal of my research was to point out the differences between excipients, with special emphasis on surfactants.

In our first experiments, creams containing *Spirulina platensis* lyophilisate were formulated using various nonionic emulsifiers and Transcutol HP as a solubilizer. The investigations have pointed out that the formulation containing SP 70 sucrose ester emulsifier and Transcutol HP has a good consistency and diffusion profile and this composition presents the highest antioxidant activity in UV radiation-induced oxidative stress on HaCaT cells. In addition, it has antimicrobial activity against the two main pathogens of acne vulgaris, *C. acnes* and *S. aureus,* and has a minimal negative effect on the viability of HaCaT cells. One of the outstanding results of our experiments with *S. platensis* algae is the development of a topical preparation containing a natural active ingredient, which offers an alternative treatment for one of the most common skin diseases, acne vulgaris, with fewer side effects and no antibiotic resistance.

In the second experiments, we formulated anti-inflammatory ointment, creams and gels containing *C. officinalis* extract in combination with diclofenac sodium and methyl salicylate to achieve the optimal therapeutic effect. Based on the rheological and the diffusion studies, the incorporation of the active ingredients into a gel formulation results in more favorable properties compared to ointment and creams, suggesting a high tissue concentration. This can provide a greater and longer-lasting analgesic and anti-inflammatory effect. To the best of our knowledge, the human clinical trial discussed in this dissertation was the first study to investigate the combination of *C. officinalis,* diclofenac sodium, and methyl salicylate in the treatment of rheumatoid arthritis. In our experiment, we were able to demonstrate that the formulation with Synthalen K gel-forming polymer can significantly reduce pain in patients as well as the degree of inflammation in the affected joint. The *C. officinalis* gel we had formulated may provide an excellent alternative treatment option for patients with rheumatoid arthritis without systemic side effects.

6. Publications related to the dissertation of the candidate

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Candidate: Liza Józsa
Doctoral School: Doctoral School of Pharmacy
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List of publications related to the dissertation

1. **Józsa, L.**, Ujhelyi, Z., Vasvári, G., Sinka, D. Z., Nemes, D., Fenyvesi, F., Váradi, J., Vecsernyés, M., Szabó, J., Kalló, G., Vasas, G., Bácskay, I., Fehér, P.: Formulation of Creams Containing Spirulina Platensis Powder with Different Nonionic Surfactants for the Treatment of Acne Vulgaris.
Molecules. 25 (20), 1-23, 2020.
DOI: http://dx.doi.org/10.3390/molecules25204856
IF: 4.411

2. Jurca, T., **Józsa, L.**, Suciu, R. N., Pallag, A., Marian, E., Bácskay, I., Muresan, M., Stan, R. L., Cevei, M., Cioară, F., Vicas, L., Fehér, P.: Formulation of Topical Dosage Forms Containing Synthetic and Natural Anti-Inflammatory Agents for the Treatment of Rheumatoid Arthritis.
Molecules. 26 (1), 1-26, 2020.
DOI: http://dx.doi.org/10.3390/molecules26010024
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List of other publications

3. Bácskay, I., Sinka, D. Z., **Józsa, L.**, Vasas, G., Ujhelyi, Z., Fehér, P., Juhász, B., Szilvássy, Z.: Formulation and investigation of turmeric extract and sodium benzoate loaded capsules. *Acta Pharm Hung. 91* (1), 11-20, 2021. DOI: http://dx.doi.org/10.33892/aph.2021.91.11-20

4. **Józsa, L.**, Fehér, P.: Amit a Spirulina algáról tudni érdemes. *Gyógyszerészet. 2020* (3), 160-164, 2020.

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