Innovative therapeutic options for patients with bladder pain syndrome

PhD thesis

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The isolation and examination of biologically active compounds program

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1. Introduction

Bladder Pain Syndrome (BPS), also known as Interstitial Cystitis (IC) is a chronic non-bacterial inflammatory disease and it affects many people worldwide. The nomenclature of BPS has undergone several changes over the last century, but the European Association of Urology guideline recommends the use of the term BPS. It is a condition with frequent urinary complaints and considerable pain, which has a major impact on the patient's quality of life. Unfortunately, the diagnosis rate is very poor. Symptoms and severity of the disease vary according to its stage. In some patients, there is only mild discomfort and pressure in the bladder associated with more frequent urination. In advanced stages, however, patients may suffer from very severe pain, sometimes permanent, accompanied by a constant urge to urinate day and night. In the absence of appropriate treatment, the disease progresses and over the years, the bladder and kidneys become constricted and fail. The disease can be accompanied by depression and impaired sexual function very often, again leading to a deterioration in the patient's quality of life. The exact cause of the development of bladder pain syndrome is still unknown, but there is evidence of a background of complaints due to insufficient production of the superficial lymph layer of the bladder. This layer is not impenetrable, but in its intact, undamaged state, it maintains a balance between inflow and outflow. The problem is caused by the loss of continuity of the glycosaminoglycan-layer with respect to the irritants in the urine. This leads to chronic chemical irritation and inflammation of non-bacterial origin (sterile inflammation) in the layers of the bladder wall. This is supported by the analysis of biopsy specimens from patients with BPS. Loss of the barrier is associated with pathophysiological, gene expression and molecular changes. Examples are blisters with Hunner lesions showing radial vascularisation with areas of epithelial ulceration covered with white fibrin in the centre. Furthermore, these lesions show diffuse and intense inflammation due to the overexpression of genes that promote inflammation. These lesions are not always present in the bladder and we have therefore distinguished between Hunner lesion BPS and non-Hunner lesion BPS. Pain is also increased by the proliferation of mast cells and the histamine they produce. There is an increase in the number of receptors at these sites, due to prolonged stimulation of pain receptors, which results in further pain intensification. As a result of these processes, connective tissue cells migrate into the oedematous tissues and the bladder loses its elasticity. This process can lead to the development of a scarred bladder over many years.

2. Aims

- Development of a national BPS treatment protocol. Our protocol covered both dietary and drug treatment aspects.
- Development of a potassium-free extended-release tablet to provide 2x1 dose per day to mitigate ongoing pain and potential side effects (e.g. reflux). Thus increasing patient adherence.
- To reduce sterile, chronic inflammation in the bladder at the cellular level, lavender and eucalyptus essential oils and their main constituents (linalool, eucalyptol) were used in T24 bladder epithelial cell line studies. Our aim was to determine the extent of the anti-inflammatory effect based on changes in mRNA expression of pro-inflammatory interleukins (IL-1β, IL-6 and IL-8). In addition, we compared their efficacy with the inhibitor ACHP (2-amino-6-[2-(cyclopropylmethoxy)-6-hydroxyphenyl]-4-(4-piperidinyl)-3-pyridinecarbonitrile), a proven NFkB inhibitor. In addition to the changes in mRNA levels, the anti-inflammatory effects of the components were also examined at the protein level.

3. Material and methods

3.1 The BPS treatment protocol was created after study for foreign association guideline

The protocol proposal for the management of bladder pain syndrome was based on international recommendations and national experience. Currently, there are no officially accepted professional recommendations for the treatment of bladder pain syndrome in Hungary. In order to develop our protocol, we consulted urological specialist to establish a safe and continuous medication supply. We made our recommendation by studying the symptoms and treatment of IC patients treated by them, as well as their medication. We also took into account the guidelines published by the Urological Societies of different countries. We requested an official authorisation for prescribing medicines beyond the indication used in the therapy of BPS (OGYÉI/64008-4/2019). In addition, to better understand the factors that positively or negatively influence the outcome of BPS, we have compiled a questionnaire on nutrition. The data obtained from the questionnaire "Impact of medications, non-medicinal products, dietary and lifestyle habits on symptoms of interstitial cystitis (bladder pain syndrome) - questionnaire survey" is intended to improve the quality of patient care. Data were collected and managed anonymously. The questionnaire was sent to 246 patients, of whom 126 participated in the survey, aged between 30 and 99 years. Data collection took place between March and May 2019. The questionnaire contained 30

questions. The questionnaire contains single- and multiple-choice questions and some short explanatory answer options. The survey was conducted under the authorization of the Health Science Council, Science and Research Ethics Committee No. 8230-2/2019.

3.2 Formulation of potassium-free prolonged-release tablets

We have set the active substance content of our own unique K^+-free formulation to 60% of the tablet weight. The main active substances of the tablets were citric acid, sodium citrate and magnesium citrate. Each active substance was powdered separately in a mortar and after sieved until a particle size of 160 μm was obtained, in accordance with the Hungarian Pharmacopoeia. Then Aerosil was added in small portion. After homogenisation, a magnesium stearate excipient was added to ensure the desired lubricant effect. In addition, a high water-binding excipient Avicel DG was used. Benecel hypromellose grades with high viscosity were used as release rate regulators: Benecel K4M PH DC, Benecel K15M PH DC and Benecel K100M PH DC. In each case, five different tablet powder formulations were prepared and subjected to different examination.

3.2.1 The rheology of granules

Flow properties of the 15 different compositions were determined according to Ph. Hg. VIII. The Hausner ratio and Carr index were determined using a Stampf volumeter. ASTM funnel was used for the outflow time determination and for calculating the angle of repose.

3.2.2 Tablet compression

A Korsch single-punch tablet press equipped with oval shape puches (dye: 7.6 mm, tablet height: 16.2 mm). Compression forces were 10 kN in all case. Average weight of the tablet was 643 ± 2.44 mg.

3.2.3 Tablet's physical properties

The tablets prepared in this way were examined according to the requirements of the VIII. Hungarian Pharmacopoeia, as follows:

- Uniformity of mass of single-dose preparation
- Friability of uncoated tablets
- Resistance to crushing of tablets
- Dissolution test of active substances
- Determination of the content of active substances

3.3 Analytical study of potassium-free prolonged-release tablets

3.3.1 Drug release testing of tablets

Rotating paddle method was used with the following test parameters: temperature 37°C, 75 rpm, dissolution media: 900 mL of pH 6.8 phosphate buffer and 2.4 mL of sample volume.

3.3.2 Determination of the active substance in the tablet

HPLC analyses of the active substance content was performed as follows: 1 mL of the sample was acidified with 20 μL of HPLC grade phosphoric acid.

After completion of the HPLC method validation, the following test parameters were applied:

- column: Lichosper C18 250 x 6 mm
- flow rate 1.2 mL/min
- injection volume 10 μL
- eluents: A: 0.05% H_3PO_4 /H_2O; (D: 10% H_2O/MeOH column wash only)
- 26°C (isocratic conditions)
- limit of detection (LOD) 0,0607 mg/L

3.3.3 Dissolution data analysis

A model-independent analysis was performed to determine the comparability of the dissolution profiles of the tested tablet composition and the value of the similarity and difference between them. Dissolution efficacy was also calculated for the average dissolution profile of all compositions. We calculated the leaching efficiency for the average leaching profile of all compounds, as well as the difference, f_1 factor and similarity f_2 factors. For kinetic modelling of drug release, dissolution data for the C/3 formulation were nonlinearly fitted with zero-order, first-order and Korsmeyer-Peppas models using Microsoft Excel.

3.3.4 Stability test of C/3 tablet

After 24 months, we examined the stability of the C/3 tablets that we formulated and tested. It was stored in a dry place, protected from light. During the stability test, all the measurements described above were repeated.

3.4 Tablet efficacy in a double-blind randomised placebo-controlled human clinical pilot study

The trial was conducted at a single location; at Rózsakert Medical Center Budapest. According to the inclusion criteria, twenty adult patients, four men, and sixteen women,

previously diagnosed IC/BPS patients on maintenance therapy were involved on a voluntary basis. The trial was carried out under a licence of the Local Committee of Research Ethics (permit number: 7074–2/2020). The endpoint of the human clinical pilot study was the determination of mean urinary pH. Whether the prolonged-release citrate tablet has an adequate alkalinizing effect compared with the administration of immediate-release citrate tablets and placebo. Due to the requirements of a placebo-controlled, double-blind clinical pilot trial, the three different tablets had to be of the same appearance and mass. Patients were given a freshly calibrated, digital, Testo-type, portable pH meter and an accurate pH measurement protocol that included step-by-step instructions. Data were registered on a website specifically designed for the clinical trial. In case of urgent questions, a telephone contact was provided to the doctor leading the clinical trial. Statistical analysis of the data was performed using Statistica (TIBCO Software Inc. (2018) Statistica (Data Analysis Software System), version 13.

3.5 Examination of the anti-inflammatory effect of essential oils on the T24 epithelial cell line

3.5.1 Purchase of essential oils and preparation of stock solutions

The lavender was collected in two phenophases of the plant: at the beginning of the flowering period (20 June, 2019) and at the end of the flowering period (18 July, 2019), and the essential oil distillation was performed at the Institute of Pharmacognosy, PTE GYTK. The location of the plant harvesting was: Bolhó village. The essential oil was extracted from the plant by water-steam distillation based on the description of the 8th edition of the Hungarian Pharmacopoeia. The essential oil content was measured with the volumetric method. The eucalyptus essential oil was purchased from Aromax Ltd. The linalool and eucalyptol standards were ordered from Sigma-Aldrich. Dimethyl sulfoxide was added to the essential oil in the preparation of the stock solutions of the essential oils. The emulsions were vortexed and diluted with phosphate buffered saline. Stock solutions of linalool and eucalyptol standards were prepared in the same way as essential oils.

3.5.2 GC-MS analysis

Before to analytical analysis, eucalyptus essential oil (10 μL) was dissolved in 990 μL n-hexane (dilution: 1:100), lavender oils (10 μL) were dissolved in 990 μL methanol (dilution: 1:100) and injected into GC-MS and GC-FID systems. Gas chromatographic measurements of lavender essential oil were performed at the University of Messina, Italy. The gas

chromatographic measurements of eucalyptus essential oil were performed at the Institute of Pharmacognosy, Faculty of Pharmacy, Semmelweis University.

3.5.3 Cell culture and treatments

T24 human bladder epithelial cells were maintained in McCoy's 5A Medium Modified supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. T24 cells were seeded in 6-well plates and cultured for 24 h before treatments. The inflammation was induced using TNFα treatment for 6 h and 24 h. TNFα concentration was determined by concentration dependence experiments followed by the analysis of proinflammatory cytokine mRNA expression. The anti-inflammatory effects of essential oils (EOs) and their main components were determined in three different experiments: a) TNFα pre-treatment for 6 h followed by 24 h of EO/standard treatment; b) TNFα pre-treatment for 24 h followed by 6 h of EO/standard treatment; and c) TNFα pre-treatment for 24 h followed by 24 h of EO/standard treatment. The EOs and standards were used at 500-fold dilution of stock solutions to determine their effect on cytokine production. In all experiments, cells treated with DMSO were used as controls. All experiments were carried out at 37°C in a humidified atmosphere containing 5% CO_2.

3.5.4 Cell viability assay

The cells were treated with essential oils and standards in 200-fold, 500-fold, 1000-fold and 2000-fold dilutions for 6 h and 24 h. Viability of the T24 cells were measured using Cell Counting Kit-8 (CCK-8) after the treatments. Cells treated with DMSO were used as a control for cells treated with essential oils, while the effect of DMSO on cell viability was used as a control for untreated cells. The absorbance of the samples was determined at 450 nm with a MultiSkan GO microplate spectrophotometer. Cell viability was expressed as a percentage of the total cell number of the corresponding control.

3.5.5 Real-time PCR

The samples were subjected to total RNA isolation using the Quick RNA mini kit. The complementary DNA was synthesized from 200 ng of total RNA following the protocol of the high capacity cDNA Reverse Transcription Kit. Gene expression was measured in 20 µL total reaction volume using a CFX96 real-time PCR system with iTaq™ Universal SYBR® Green Supermix. The Livak (2-ΔΔCt) method was used for relative quantification using a Bio-Rad CFX Maestro software. The gene expression levels obtained in the samples were plotted against β-actin levels.

3.5.6 Enzyme-linked immunosorbent assay (ELISA) measurements

After each treatment of cells, the culture supernatant of control and treated cells was collected and kept at −80°C until measurements. The concentration of IL-8 secreted in the culture media was determined using human IL-8 ELISA kit following the manufacturer's instructions.

3.5.7 Statistical analysis on the anti-inflammatory effects of essential oils

Statistical analysis was performed using SPSS software. Statistical significance was determined by one-way ANOVA followed by Tukey's HSD post hoc test. Data are shown as mean±standard deviation (SD). Statistical significance was set at p value < 0.05. Each assay was carried out in triplicate in each independent experiments (n=3).

4. Results and conclusions

4.1 The BPS treatment protocol was created after study for foreign association guideline

4.1.1 Treatment protocol for BPS

In patients with mild symptoms in the early stages, an IC diet, plenty of fluids and urinary alkalinisation may be recommended to reduce the effects of urinary irritants. These may be accompanied by oral GAG-layer regenerating drugs such as chondroitin sulphate and pentosan polysulphate sodium. Initial treatment may be supplemented, if necessary, with anti-inflammatory drugs, antihistamines and anxiolytics.

In more advanced stages of moderately severe symptoms, combination therapy, which is the simultaneous use of oral and topical treatment, is most effective. Intravesical solutions containing the main components of the GAG layer are administered into the bladder by specialists. This treatment should lead to regeneration of the GAG layer and consequently to a reduction in the inflammation of the bladder wall.

In severe stages, or if the condition worsens despite treatment, surgical removal of the affected areas is necessary: by transurethral laser or electrocoagulation. In the terminal stage, a gall bladder may develop. In this case, cystectomy is the last resort. If the outpatient therapy is not sufficient, symptomatic treatment may be necessary. This means strong analgesics, antidepressants, muscle relaxants, antihistamines and anxiolytics.

4.1.2 Comparing international guidelines on medication

The world's leading urological societies: the American (AUA), British (RCOG), European (EAU), Canadian (CUA) and Japanese (JUA) have published five main guidelines that set

out grades of recommendation. A review of the active substances included in our protocol led to the following conclusions among the guidelines.

1. Oral pentosan polysulphate is recommended for the treatment of milder symptoms, as it strengthens the GAG and reduces damage to the urothelium. The EAU and RCOG guidelines have classified the drug as a level A recommendation. The AUA and JUA have level B evidence in this regard. The level of evidence for the CUA directive is D.

2. Heparin is included in our protocol proposal. The recommendation for intravesical heparin in the EUA guideline is less recommended than in the RCOG guideline (D). The evidence for the AUA and CUA protocols is level C, as is the evidence in the JUA guideline.

3. The level of evidence for the recommendation on hyaluronic acid is not clearly defined. In the ROCG guideline, the level of evidence is B and the AUA protocol does not include this active substance. In the JUA and CUA guidelines, the level of evidence is C. The protocol we recommend includes it because it may be important for GAG layer repair: it reduces membrane permeability, which is necessary for GAG layer regeneration.

4. Both oral and intravesical chondroitin sulphate are included in our proposal, as they are a component of the GAG layer. Its use is supported by positive experience, as it is included in a number of formulations marketed in the UK and EU (e.g. Uracyst®, iAluRil®). However, the level of recommendation is also not uniform. Indeed, there is weak EAU guideline recommendation and level II evidence for intravesical chondroitin sulphate. The level of evidence is level D in the ROCG and CUA guidelines and level C in the Japanese guideline.

4.1.3 The results of the dietetic questionnaire survey

After the answers to the dietetic question of the questionnaire were evaluated, the following results were obtained. The difficulty in diagnosing the disease was also reflected here, with 37% of patients being diagnosed after 4 years, and in the most fortunate cases 10% of patients within 1 year. The most common complaints that patients consulted their doctor with were bladder pain, frequent urge to urinate, urethral pain, vaginal pain and bloody urine. The 60% of the medications taken by patients before diagnosis were antibiotics. The 19% were taking painkillers and 14% were taking herbal preparations or combinations of these. The most common medication, prescribed by the urologist, was an intravesical solution called a special bladder cocktail, which 51% of those surveyed had received. The other mainstay of treatment is pentosan polysulphate, which is considered effective, as well as non-steroidal anti-inflammatory drugs and painkillers. In addition, 24% of patients were also

taking other medicines such as antibiotics and urine alkalis. More than 50% of the patients surveyed had other chronic conditions. Most of these patients had high blood pressure and high cholesterol, but many also had allergies and reflux. 82% of patients changed their eating habits after being diagnosed with the disease. In general, they switched to diets recommended for BPS patients. Among dairy products, milk is recommended by the regulations. In contrast, 23% of former milk consumers surveyed no longer consume milk at all. A similar decrease was also observed for yoghurt, cottage cheese, sour cream and long-ripened cheeses. In the case of coffee, black and green teas and energy drinks, the majority of patients had regularly consumed one of these products before being diagnosed with the disease. After diagnosis, this figure fell to 21%. In contrast, consumption of decaffeinated coffee increased by 32%. In addition, we also assessed which drinks increased symptoms. Patients also mentioned alcoholic drinks, orange juice, blackcurrant juice and carbonated soft drinks. Regarding alcohol consumption habits, 60% of patients said they did not drink any alcohol. We also asked patients about their vegetable consumption habits. Tomato consumption decreased most significantly (by 60%) after diagnosis. In addition, consumption of onions, green onions, beans and potatoes also decreased. In addition, spinach, broccoli and pumpkin were also mentioned as vegetables that increased their symptoms. We also asked about the consumption of some popular fruits (apples, bananas, apricots, peaches, watermelons, blueberries). The questionnaire survey showed that consumption of each of these had fallen by about half following diagnosis. In addition, some patients said that citrus fruits, grapes, blackberries, raspberries, cherries and pears also increased their symptoms. International recommendations suggest that spicy and highly spiced foods can worsen patients' symptoms. Accordingly, the majority of people surveyed (66%) do not eat these foods. 64% of our patients take dietary supplements and vitamins. 17% use herbal products regularly and 49% only rarely. The most common were lemongrass (37%), cranberry (29%) and bearberry (25%).

4.2 Formulation of potassium-free prolonged-release tablets

4.2.1 The results of rheological and pre-dissolution examination

Based on the rheological results and preliminary examination, only 3 additional tablets were further investigated, which were tablets of formulation A/3; B/3 and C/3. The rheological results of the selected granules were as follows. For the composition A/3, the Hausner ratio was 1.15, Carr index 13.72 and the angle of repose 43.83°. For composition B/3, the Hausner ratio was 1.17, Carr index 15.34 and the angle of repose 45.83°. The rheological results for

the C/3 powder blends were as follows: Hausner ratio 1.17, Carr index 15.28 and angle of repose 44.77°. The preliminary drug release test was performed on the 7 tablets by three parallel measurements.

4.2.2 Physical parameters of tablets

The results obtained for the determination of the individual and average weights are in accordance with pharmacopoeial specifications. For N = 30 tablets the maximum deviation was 5%. Uniformity of active ingredient content: min 372.4 mg; max 422.6 mg; mean of 6 tablets: 397.83± 2.99 mg. Tablet attrition loss was also adequate. For N = 20 tablets, it was 0.88% ± 0.01, which met the pharmacopoeial criteria. The mean value of fracture toughness (n = 20 tablets) was 182 N±4.3.

4.2.3 In vitro drug release

In vitro dissolution data of all compositions were compared, composition C/3 were found to release its API content in the most balanced manner. An average of 28.33% of the citrate salts dissolves in 1 h. Subsequently, 54.64% of the active ingredient is dissolved in 3 h, and after 6 h, 80.83% of the active ingredient is released from the matrix tablet. Composition C/3 was compared in a pair-wise way to composition A/3 and B/3, difference and similarity factors. When the mechanism of drug release is elucidated, the coefficient of determination of the fit for the First-order model was found with the highest value. In support of first-order kinetics, compared to the immediate-release tablet, the release rate of the prolonged-release tablet (C/3) is slower and more uniform.

4.2.4 Stability test of C/3 tablet

The parameters of the tablet after 2 years were: length 16.2 mm, oblong tablet width: 7.6 mm. Mean value of crushing strength (n = 20 tablets): 167.9 N ± 3.3. The average mass was 625.2 ± 1,4 mg. Friability was 1.02% ± 0.02. Based on these results, the average weight of a tablet decreased by 2.78% and the crushing strength by 7.75%. C/3 tablets stored for 24 months were analysed and compared to the initial dissolution data. Dissolution efficacy was found to be 89.41%. Model fitting results showed no alteration even after 24 month, First-order release model was found with the highest value for determination coefficient, namely R2 = 0.9743. Difference and similarity was also assessed for C/3 tablets regarding their dissolution. Value of difference and similarity factor was 8.34 and 59.94.

4.3 Efficacy of the tablet in a double-blind randomised placebo-controlled human clinical pilot study

The weekly average of the urine pH showed no significant difference between the washout period and the placebo. Both the immediate release citrate tablet and prolonged-release citrate tablet caused a significant rise in the urine pH (weekly average) compared to the weeks when no active alkalizing compounds were administered (washout and placebo). The weekly average of the urine pH showed no significant difference between the two alkalizing tablets. Considering that prolonged-release tablet contained 22% less active ingredients, this result can refer to the fact that the biological utilization of the prolonged-released tablet was more effective.

4.4 Examination of the anti-inflammatory effect of essential oils on the T24 epithelial cell line

4.4.1 Effect of essential oils and standards on T24 human bladder epithelial cell viability

The results showed that DMSO did not affect cell viability. For T24 cells, the following changes in viability were observed. Lavender oils and linalool at 200x dilution did not cause a decrease in cell number at 6 hours. During the 24 h treatments, linalool was found to be highly toxic to the cells, while lavender oils caused only mild toxicity. At 500-fold, 1000-fold and 2000-fold dilutions, cell viability was no longer significantly affected by either linalool or lavender oils at 6 and 24 hours. In the case of eucalyptus oil and eucalyptol, none of the test substances were toxic to cells at any dilution during 6 h treatments. Toxicity was measured only in the 24-hour studies, with the 500-fold dilution being the least harmful to cells.

4.4.2 Effects of lavender essential oils and linalool standard on mRNA expression of IL-1β, IL-6 and IL-8 after TNFα pre-treatment of T24 cells

A significant reduction in IL-8 mRNA expression was observed for all oils and standard treatments. In case of IL-8, using 6 h essential oil treatment after 24 h of TNFα pre-treatment, essential oil distilled from lavender at the end of flowering period was found to be the most effective. The change of IL-6 was only considered to be effective the linalool standard. For the short treatment (6 h) after TNFα 24 h pre-treatment, the highest IL-1β expression alteration was measured for the essential oil obtained from the end of flowering. In case of IL-6 mRNA levels, the most effective treatment was 24 h essential oil treatment after 6 h of TNFα pre-treatment. Based on changes in IL-8 the linalool standard proved to be more effective than EOs. A decrease in IL-1β mRNA expression was observed 24 h after 6 h TNFα pre-treatment with linalool standard and end of flowering essential oil (24 h). For the long

treatment (24 h) after TNFα 24 h pre-treatment, the greatest change in mRNA expression was observed for IL-8. In this case, the most effective was the beginning of flowering lavender essential oil. In the case of IL-6 again, the effect was most pronounced for linalool. The greatest reduction in IL-1β expression was seen with the linalool standard. Based on the IL-1β mRNA expression, this treatment was proved to be the least effective in this case.

4.4.3 Effects of eucalyptus essential oil and eucalyptol standard on mRNA expression of IL-1β, IL-6 and IL-8 after TNFα pre-treatment

Changes in IL-8 chemokine expression were as follows. During 24 h of treatment after 6 h of TNFα pre-treatment, eucalyptus oil was more effective than the standard. For the short treatment after 24 h TNFα pre-treatment, the eucalyptol standard was found to be better than eucalyptus oil, while for the 24 h treatments, essential oil was found to be more effective. Regarding the change in IL-6 expression, eucalyptus oil was better in reducing IL-6 expression in 24 h treatments after 6 h TNFα pre-treatment. Similar results were obtained for the 6-h treatment after 24 h TNFα pre-treatment. Only after 24 h of treatment did eucalyptol show greater efficacy compared to essential oil. The change in IL-1β mRNA expression after 24 h of treatment following 6 h of TNFα pre-treatment also showed a more pronounced effect of eucalyptus oil, as was observed for the other three cytokines tested. However, for both the 6-hour and 24-hour treatments following TNFα pre-treatment, the eucalyptol standard was more effective.

4.4.4 Comparing the effects of lavender oils and linalool with ACHP treatment

The effect of lavender essential oils and linalool were compared to the efficacy of ACHP treatment, that is inhibits the entire NFκB pathway. After TNFα 24 h pre-treatment, IL-6 and IL-8 mRNA expressions decreased most after 6 h of ACHP treatment. It should be highlighted that lavender essential oil prepared at the end of flowering showed better result than the ACHP inhibitor for IL-8. The 24 h ACHP treatment following 6 h TNFα pre-treatment significantly reduced IL-6, IL-8 and IL-1β expression. Compared to the ACHP treatment, better results for IL-8 were obtained with linalool and EO from end of flowering period. Moreover, for IL-6, essential oil from end of flowering was more effective than ACHP treatment. In case of IL-1β, the alteration in mRNA expression in response to linalool treatment and end of flowering essential oil was more beneficial than the ACHP NFκB inhibitor treatment. At 24 h TNFα pre-treatment and 24 h ACHP treatment, both IL-6 and IL-8 expressions were reduced in almost equal extent. Essential oils and linalool were not effective compared to ACHP treatment in this case.

4.4.5 Comparing the effects of eucalyptus oils and eucalyptol with ACHP treatment

Compared to the DMSO control, the NFκB inhibitor ACHP significantly reduced the expression of all cytokines tested. The 24 h TNFα pre-treatment and 6 h ACHP treatment reduced IL-6 expression the most, followed by IL-8 and IL-1β. Neither eucalyptus oil nor eucalyptol was more effective than the inhibitor treatment for any of the cytokines. Comparing essential oil and eucalyptol, oil was more effective for IL-8, but eucalyptol was more effective for IL-6 and IL-1β. For treatments 24 h after 6 h of TNFα pre-treatment, we again observed the greatest reduction in expression for IL-6, followed by IL-8 and IL-1β. A more effective treatment than ACHP was obtained after the application of eucalyptus oil for both IL-8 and IL-6. For 24-hour ACHP treatments following 24-hour TNFα pre-treatment, nearly equal decreases in IL-8 and IL-6 expression were observed. For IL-8 and IL-6, eucalyptus oil was more effective, whereas for IL-1β, the decrease in expression was much smaller compared to the control and eucalyptol was more effective.

4.4.6 IL-8 enzyme-linked immunosorbent assay (ELISA) measurement

The most promising inhibitory effect of the examined essential oils and their main compounds was found in case of IL-8 chemokine. Significant reductions were measured 24 h after short term TNFα pre-treatment and 6 h after long term TNFα pre-treatment in response to ACHP inhibitor treatment. Lavender essential oils significantly reduced IL-8 protein levels 24 h after 6 h of TNFα pre-treatment. Moreover, the EO distilled from the beginning of flowering was more effective. Treatments with EO of lavender or linalool standard after 24 h TNFα pre-treatment were not capable to decrease the IL-8 protein levels. In the case of 24h eucalyptus oil and eucalyptol standard treatments, a measurable reduction was observed at 24 hours after TNFα pre-treatment.

5. Summary and new conclusions

The work focused on providing the most complete information on the current information on bladder pain syndrome, the available therapies and to address the gaps in the therapeutic areas. As bladder pain syndrome is a poorly understood, difficult to diagnose disease that is likely to affect a large number of people worldwide. The underlying cause of the disease is inadequate production of the superficial lymph layer. In the absence of this layer, which is mostly composed of glucosaminoglycan molecules, substances in the urine cause irritation, which leads to a significant deterioration in the quality of life of the patient (frequent urge to urinate, severe pain when urinating, etc.).

Our work has led to the following results:

1. A protocol for the management of medication and diet in patients with bladder pain syndrome has been developed. Where necessary, OGYEI approval for prescribing medicines beyond the indication.

2. We have developed a new innovative tablet. It is potassium-free and does not irritate the bladder mucosa. Moreover, it provides a continuous alkalinisation of the urine chemistry thanks to the prolonged-release of the active substance. The double-blind, placebo-controlled, randomised pilot study clearly demonstrated that our innovative product significantly increases urine pH compared to the control group.

3. In a sterile inflammatory T24 human uroepithelial model, lavender essential oils were found to be more effective than eucalyptus essential oil. Furthermore, based on the amount of IL-8 proinflammatory cytokine secreted by T24 cells, we concluded that lavender essential oils were more effective than treatment with the ACHP inhibitor. When comparing the two lavender essential oils, lavender essential oil collected and distilled beginning of flowering period was more effective than essential oil obtained from end of flowering.

In conclusion, we have made important progress in the healthcare of patients with bladder pain syndrome. Our medication and dietary protocol is already being used by several urologists in Hungary with excellent results. Our protocol provides easier symptom recognition and transparency of drug therapy. Our dietary recommendations contribute greatly to the reduction of pain in patients and thus to the improvement of their quality of life. Our innovative extended-release formulation is effective in raising urinary pH and has been proven to alleviate the symptoms of patients suffering from bladder pain syndrome (urinary pain, urinary urgency, etc.). And thanks to the first-order release of the active ingredient, dosing 2x daily has been shown to be sufficient, contributing to an effective increase in patient adherence. To achieve and maintain a pH neutral urine, an increase in the daily dosage to 2x2 is recommended. This would provide a more effective therapy to achieve complete symptom relief in patients with bladder pain syndrome. Furthermore, based on the 2-year stability studies of the tablet, we can say that our product is stable and meets the prescribed requirements. Our newly developed product is currently under domestic production. Our research has also included testing the anti-inflammatory effects of lavender and eucalyptus essential oils and their main constituents in a sterile inflammatory model in a T24 human uroepithelial cell line. The conclusion of our results obtained here is presented in section 3, which suggests that lavender essential oils, after proper formulation, may be

suitable for use as adjunct to intravesical therapy, as their anti-inflammatory effects may well complement GAG-regenerative therapy in the bladder.

6. List of publications

6.1 Articles related to the thesis

Adrienn Horváth, Gábor Vasvári, Sándor Lovász, Györgyi Horváth, Péter Birinyi: Formulation and examination of a new urine alkalizing tablet for the symptomatic treatment of bladder pain syndrome. Journal of Drug Delivery Science and Technology 2022, 74, 103537
IF:5,062 DOI: 10.1016/j.jddst.2022.103537

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6.2 Publications not related to the thesis

Edina Pandur, Giuseppe Micalizzi, Luigi Mondello, **Adrienn Horváth**, Katalin Sipos, Györgyi Horváth: Antioxidant and anti-inflammatory effects of thyme (*Thymus vulgaris* L.) essential oils prepared at different plant phenophases on *Pseudomonas aeruginosa* LPS activated THP-1 macrophages. Antioxidants 2022,
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Edina Pandur, Alex Balatinácz, Giuseppe Micalizzi, Luigi Mondello, **Adrienn Horváth**, Katalin Sipos, Györgyi Horváth: Anti-inflammatory effect of lavender (*Lavandula angustifolia* Mill.) essential oil prepared during different plant phenophases on THP-1 macrophages. BMC Complementary Medicine and Therapies 2021, 21 (1) 287.
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Eszter Csikós, **Adrienn Horváth**, Kamilla Ács, Nóra Papp, Viktória Lilla Balázs, Marija Sollner Dolenc, Maša Kenda, Nina Kočevar Glavač, Milan Nagy, Michele Protti, Laura Mercolini, Györgyi Horváth, Ágnes Farkas: Treatment of Benign Prostatic Hyperplasia by Natural Drugs. Molecules 2021, 26 (23):7141.
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Horváth Adrienn, Ács Kamilla: Fototerápia helyzete, elfogadottsága, az orvosi alkalmazása Európában és Magyarországon. Terápia Natura 2020, ősz, 15-16

Birinyi Péter, **Horváth Adrienn**: A helyes vitamin-, nyomelem- és ásványianyag-bevitel a várandósoknál. GYÓGYSZERÉSZET 2019, 63, 716-722.

Birinyi Péter, **Horváth Adrienn**: A várandósgondozásról-2: Táplálkozási ajánlások várandósoknak. GYÓGYSZERÉSZET 2019, 63, 609-61

Birinyi Péter, **Horváth Adrienn**: A várandósgondozásról-1: Teratogén és fetotoxikus gyógyszerek. GYÓGYSZERÉSZET 2019, 63, 601-608

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7. Abstracts

7.1 Oral presentations

Horváth Adrienn, Birinyi Péter: Advanced pharmaceutical care for interstitial cystitis. Congressus Pharmaceuticus Hungaricus XVI. Debrecen, Hungary, 10-12 September 2020 ACTA PHARMACEUTICA HUNGARICA 2020, 90, 60-60

7.2 Poster presentations

Horváth Adrienn, Pandur Edina, Sipos Katalin, Horváth Györgyi: Levendula és eukaliptusz illóolajok gyulladáscsökkentő hatásának vizsgálata hólyagfájdalom szindróma in vitro sejtkultúrás modelljében, T24 sejteken. XXV. Tavaszi Szél Konferencia, Pécs, 2022.05.06-05.08.

7.3 Other poster presentations

Horváth Adrienn, Pandur Edina, Jánosa Gergely, Pap Ramóna, Sipos Katalin: Interaction of dithiothreitol-induced unfolded protein response and vitamin D in a neuronal cell model. Annual Meeting of the Hungarian Biochemical Society, 25-27 August, 2022, Pécs Hungary

Jánosa Gergely, Pandur Edina, Pap Ramóna, **Horváth Adrienn**, Sipos Katalin: A D-vitamin selejtfehérje válaszra (unfolded protein response) gyakorolt hatása az idegrendszer vonatkozásában. XXV. Tavaszi Szél Konferencia. Pécs, 2022.05.06-05.08.

Böszörményi Andrea, Szögi-Tatár Bernadett, **Horváth Adrienn**, Balázs Viktória Lilla, Kovács Judit, Schneider György, Horváth Györgyi: 100%-ban tiszta és természetes: Mi rejlik egy illóolajos üvegben? METT25 a Magyar Elválasztástudományi Társaság jubileumi konferenciája. Egerszalók, 2021.10.18.-2021.10.20. Magyar Elválasztástudományi Társaság: P-08. (2021). ISBN: 9786155270666

Birinyi Péter, Tinta Anikó, Árvai István, Hubay Zsuzsa, Vukmann Nikolett, **Horváth Adrienn**: Drug developments for rare diseases. Hungaricus XVI. Debrecen, Hungary, 10-12 September 2020 ACTA PHARMACEUTICA HUNGARICA 2020, 90, 48-49

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