SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

Formulation and investigation of oral drug delivery systems containing herbal active ingredients

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1. Introduction and aims

The therapeutic use of herbal ingredients is present in every period of medicine. Despite of the wide variety of synthetic and biopharmaceuticals today, there is also a demand for phytopharmacons, partly because of the positive view of natural therapy, and because of the several advantages of their use.

Herbal drugs are cheap and easily accessible, they can be used for prevention and therapy, and in case of plants containing multiple active ingredients, sinergist effects can occur, with preferable side-effect profile.

However, modern, evidence-basec therapeutic practice has several requirements toward herbs. Standard and characterized active ingredient content and quality assurance not always can be ensured, and the physicochemical properties of phytopharmacons, like water solubility, permeability and stability are frequently poor, which leads to low bioavailability.

Among the pharmaceutical researches of the last decades, we can find several investigations aiming to fullfill these requirements and improve the bioavailability of herbal ingredients by modifying their physicochemical parameters or by incorporating them into modern drug delivery systems.

During my PhD research, my aim was to enhance the oral bioavailability of two herbs by different pharmaceutical formulations. In the first part, fenugreek (*Trigonella foenum-graecum*) was used, which is mostly investigated for its antidiabetic effects. It demonstrates hypoglycaemic properties through the inhibition of carbohydrate-digesting enzymes, and can be used in the prevention and therapy of diabetes and insulin resistance. Its antioxidant, gastrointestinal hepatoprotective and immunmodulatory effects are also described. The aqueous solubility, permeability and stability of fenugreek compounds ranges from poor to good. Several studies engaged in the incorporation of fenugreek seed components into modern drug delivery system formulations, but in most cases, these are used as excipients. In my research self-emulsifying drug delivery systems (SEDDS) containing standardised extract of fenugreek seed were formulated, and their physical parameters and antioxidant properties were investigated. Permeability tests were carried out to compare the gastrointestinal absorption of the main herbal components from the native extract with the SEDDS systems. The effect of the SEDDS systems on cell viability was also examined by the monolayer integrity follow-up after the permeability tests and MTT assay. Eventually, the dissolution of the SEDDS systems from hard gelatine and HPMC capsules was investigated.

In the second part, silymarin from milk thistle (*Silybum marianum*) was used. This flavonolignan complex mainly used as a hepatoprotective agent, for the prevention and treatment of cancer, and for gastrointestinal problems, among several other effects. Its solubility and intestinal absorption are poor. Its bioavailability was increased by formulating sustained release hydrophilic matrix tablets using different types of Carbopols. This technology was combined with complexation with different beta-cyclodextrine derivatives, to enhance water solubility. Physical parameters of the tablets were investigated to ensure the fulfillment of the requirements of the European Pharmacopoeia in terms of weight uniformity, friability, and hardness. Dissolution studies of Carbopol based hydrophilic matrix tablets containing silymarin and cyclodextrin-complexed silymarin were carried out in artificial intestinal fluid, artificial fluid and in changing medium for the better modelling of human gastrointestinal conditions. By the evaluation of the studies the formulations with the most proper pharmacon release were chosen, and they were compared with a commercial silymarin product. The effect of the formulation with the optimal results on cell viability was also studied.

2. Materials and methods

2.1. Preparation of fenugreek extract

The extraction was carried out in the Department of Botany, University of Debrecen. Fenugreek seeds of pharmaceutical quality were ground at 10,000 rpm for 2 min. A total of 60.0 g of seeds was extracted with 2400 mL of boiling methanol for 60 min, filtered, and evaporated to dryness in a rotary evaporator. This resulted in 13.38 g of dry extract. Thereafter, the dry extract was re-dissolved in 30.00 mL PBS and sterile filtered on a 0.22 um PES membrane. The sterile extract was stored at 4 °C before use and used within 48 h of preparation. The concentrated liquid extract in PBS contained 7.833 mg/ml trigonelline and 8.258 mg/mL 4-hydroxyisoleucine.

2.2. Formulation of SEDDS systems

Isopropyl myristate was purchased from Merck (Darmstadt, Germany). Transcutol HP, Labrasol, Kolliphor RH40 and Capryol 90 were obtained from Gattefossé (Saint-Priest, France).

The formulation method was the generally used procedure for the preparation of SEDDS systems. The oily phase isopropyl miristate, nad the emulgents and co-emulgents Transcutol HP, Labrasol, Kolliphor RH40 and Capryol 90 were mixed well in a plastic tube. Concentrated liquid fenugreek extract and PBS was added, then the mixture was mixed with gentle vortexing until we achieved a visually homogenous appearance. All the experiments were performed immediately after preparation. We formulated 5 different compositions of the liquid extract, the SEDDS excipients and PBS. One of them contained native fenugreek extract, two of them fenugreek extract SEDDS formulation, and two of them were only the excipients of the former SEDDS systems, without extract.

2.3. Particle size analysis

Particle size analysis of SEDDS systems were carried out using a Malvern Zetasizer Nano ZSP, by dinamic light scattering method. 1 ml of the sample SEDDS composition was diluted with 900 mL distilled water. The samples were allowed to equilibrate for 5 min at 25 °C before performing 5 measurements. The refractive index and absorption values of polystyrene latex were used.

2.4. Zéta-potenciál analízis

The zeta potential analysis of SEDSS systems was carried out using a Malvern Zetasizer Nano ZSP. 1 mL of the sample SEDDS composition was diluted with 900 mL distilled water. The samples were allowed to equilibrate for 5 min at 25 °C before performing 5 measurements.

2.5. DPPH antioxidant assay

The antioxidant capacities of the compositions were determined by DPPH (2,2-Diphenyl-1-picrylhydrazyl) assay, which is a standard, simple colorimetric method to determine the inhibition of reactive oxidative species. A serial dilution of the SEDDS formulations and the positive control (1 mg/ml ascorbic acid solution) was prepared, then 1 ml of the samples was added to 2 ml 0.06 mM DPPH reagent (Merck KGaA, Darmstadt, Germany). After reacting with antioxidant agents, the purple DPPH reagent transforms into a yellow compound in a concentration-dependent way, and the absorbance was measured with a Shimadzu UV-1900 spectrophotometer at the 517 nm wavelength.

2.6. Caco-2 cell line

Caco-2 human adenocarcinoma cell line was obtained from the European Collection of Cell Cultures (ECACC, Public Health England, Salisbury, UK). The cells were maintained in plastic cell culture flasks in culture medium (Dulbecco's modified eagle's medium) in a humified atmosphere of 5% CO2 at 37 °C. The tests were per-formed using cells in passages 20–40.

2.7. Superoxide dismutase (SOD) antioxidant assay

The Superoxide dismutase enzyme catalises the degradation of superoxid species, therefore it is an important part of an antioxidant defense mechanism in almost every cell exposed to oxygene. The Caco-2 cells were seeded on 12-well plates at a density of 100,000 cells/well and grown in a CO2 incubator at 37 °C for six days. The cell culture medium was removed, and 1 mL 100× dilution of each composition was added to the cells, then incubated at 37 °C for 1 h. A 100× dilution of 1 mg/mL ascorbic acid was used as the positive control, while PBS was used as the negative control. The SOD activity of the supernatant was analyzed

using assay kits from Cayman Chemicals according to the manufacturer's instructions (Cat. 706002, Cayman, Ann Arbor, MI, USA). All experiments were performed in triplicate.

2.8. Permeability test

Caco-2 cells form monolayer with tight junctions, therefore they can be used as a model of paracellular absortpion. By expressing several transport and efflux proteins they can also model different transcellular absorption process.

For the permeability assay, Caco-2 cells were seeded on 24-well polycarbonate filter inserts at 80,000 cells/insert. The culture medium was replaced with fresh every 3–4 days. Trans-epithelial electronic resistance was measured using a Millicell ERS-2 voltohmmeter with a chopstick electrode pair. All cell monolayers presented TEER values between 800 and 1000 Ω cm2.

The inserts were placed into the wells of a fresh plate. The permeability assay was commenced with the addition of 400 µl of the sample solution to the apical chambers of the inserts. A 50 µl aliquot was taken from the basal chamber containing PBS immediately, and at 15, 30, 60, 120 and 240 min.

The sample solutions used for the permeability assay were PBS (as blank) and the five studied compositions. Each sample solution was tested on 4 inserts in parallel.

The samples were processed in the Department of Botany by LC-MS analytics.

2.9. Cell monolayer integrity follow-up after the permeability tests

The cell monolayer integrity was monitored using follow-up TEER measurements. During the permeability test, tight junctions can loose, which leads to decreased TEER values. Cell layer integrity is however quickly restored between viable cells. The TEER values measured before the test were recorded, and after the 2 hour assay they were measured again with the samples replaced with fresh medium. 2 hours and then 24 hours later the TEER values were measured again. The cells were treated with Triton-X-100 as a positive control (Sigma-Aldrich, Budapest, Hungary).

2.10. MTT cytotoxicity test

The MTT method was used for the cytotoxicity assay to determine the viability of Caco-2 cells after the treatment with the composition samples. MTT is a yellow molecule which is transformed by the mitochondrial enzymes of viable cells to purple formazan. The formation of intracellulat formazan can be measured by colorimetry. The cells were seeded at a density of 10,000 cells per well on flat-bottom 96-well tissue culture plates, and were allowed to grow for 7 days in a humified atmosphere of 5% CO2 at 37 °C. The cytotoxicity assay was initiated with the removal of the cell culture medium and the washing of the cell. The sample solutions were added for a 30 min incubation period, then were removed. A further 3 h of incubation in cell culture medium containing MTT (0.5 mg/ml) followed (Sigma-Aldrich, Budapest, Hungary). The purple formazan crystals produced by cellular enzyme activity were dissolved from the bottom of the plate in a 25:1 isopropanol:hydrochloric acid solution. The absorb-ance was measured with a FLUOstar OPTIMA Microplate Reader at 570 nm against a 690 nm reference. Cell viability was expressed as the percentage of the negative controll cells treated with PBS.

In the first test, cell viability was investigated after treatment with the SEDDS compositions. A blank PBS treatment was used as a negative control, and Triton X-100 treatment as the positive control. A serial dilution was prepared from the compositions in 100, 200, 300, 400, 500, and 1000-fold, and was added to the cells.

In the second test, a serial dilution of the 4 Carbopols was prepared by dissolving 150 mg polymer in 900 ml distilled water, then added to the cells. A blank PBS treatment was used as a negative control.

In the third test, the dissolution samples of the selected tablet formulation was added to the cells. A blank PBS treatment was used as a negative control.

2.11. Modified dissolution test of antioxidant agents from capsules

A modified dissolution test was carried out with the SEDDS compositions, the method was based on the experiments of **Li et al.** and **Kalantari et al.** Hard gelatin and HPMC capsule shells (Capsugel, Inc., Morristown, NJ, USA) were filled with 1 ml of each composition. Three capsules were put in a medium of 100 ml distilled water at room temperature and stirred at 120 rpm. A 1 ml aliquot was taken from the medium at 10, 20 and 30 min in the case of the hard gelatin capsules, and at 15, 30, 45 and 60 min in the case of the HPMC capsules.

The samples were added to 2 ml 0.06 mM DPPH (2,2-Diphenyl-1-picrylhydrazyl) reagent, and the absorbance was measured with a Shimadzu UV-1900 spectrophotometer on 517 nm wavelength.

2.12. Silymarin

Silymarin powder from Silybum marianum seeds was prepared according to **Kahol et al.** at the Department of Applied Chemistry, University of Debrecen. The the milk thistle seeds were cooled and powdered, defatted. The seed powder was extracted with acetonitrile, the obtained silymarin was concentrated under vacuum, dried and filtered. The silymarin powder did not contain any solvent residue. The same bioactive flavonolignans were determined as in the standards with the help of HPLC-MS method.

2.13. Tablet formulation

4 different types of Carbopol were used as the base for hydrophilic matrix tablets:áltam fel: Carbopol 71G, Noveon® AA-1 Polycarbophil USP, Carbopol 974 PNF, and Carbopol 971P, purchased from The Lubrizol Corporation (Wickliffe, Ohio, USA).

4 different beta-cyclodextrin derivatives were used for the complexation of silymarin: 2-Hydroxy)propyl-β-cyclodextrin (DS~3±1), Heptakis(2,6-di-O-methyl)-β-cyclodextrin, Methylated β-cyclodextrin (DS~12), and Random Methyl-β-cyclodextrin (DS~12) were obtained from CycloLab R&D Ltd. (Budapest, Hungary).

Altogether 20 different tablet compositions were prepared of the 4 polymers and the 4 polymers combined with the 4 complexing agents.

For tableting excipients, Mg-stearate, talc, calcium-phosphate dibasic (Hungaropharma Zrt., Hungary), and Ludipress® (BASF, Ludwigshafen, Germany) were used.

Each product contained 70 mg silymarin as active ingredient. Silymarin-cyclodextrin complexes were made by physical mixture method: the drug and the different β-CD derivatives were weighted accurately in the required molar quantites (1:1), and mixed together in a mortar. 150 mg of the Carbopols were used in each tablet. As excipients of tablet pressing, 15 mg of Mg-stearate, 5 mg of talc, 10 mg of dibasic Ca-phosphate, and the amount of Ludipress required for the total weight of 500 mg per tablet. The excipients ensured the glidant, lubricant, anti-

adhesive and binding effects. Tablet ingredients were homogenized in mortar after being measured. For compressing, manual bench-top tablet press was used. Compressing force was 50 N.

2.14. Pharmaceutical tests (Ph. Eur. 9.)

2.14.1. Weight uniformity

For the test of tablet weight uniformity, 20 tablets were measured and the average weights were calculated. The deviation of the tablet weights from the average were calculated in percentage.

2.14.2. Friability

Tablet friability was measured using an Erweka TA40 friability tester (Heusenstamm, Germany). After carefully dedusted, the sample of 20 tablets was accurately weighed, then it was put in the drum of the tester. After 100 rotations of the drum, the loose dust was removed from the surface of the tablets and the tablets were weighed again.

2.14.3. Hardness

Tablet hardness was measured using an Erweka TBH30M tablet hardness tester (Heusenstamm, Germany). We investigated the breaking force of the sample of 10 tablets and averaged the results. All the methods and equipment used for pharmaceutical tests are in accordance with the European Pharmacopoeia 9th Edition.

2.15. Dissolution tests of matrix tablets

Dissolution studies were conducted using Erweka DT 800 Dissolution Tester (paddle method) (Heusenstamm, Germany).

In the first test silymarin dissolution was investigated from Carbopol based hydrophilic matrix tablets in artificial intestinal fluid. The medium contained 6,8 g KH_2PO_4 per liter. 0,1 M NaOH solution was used for adjusting pH to 6.8, according to Ph. Eur. 9. The chemicals were obtained from Hungaropharma Zrt. Temperature of the medium was 37±0.5 ºC and it was constantly stirred through the tests, the paddle rotation speed was 100 rpm. Each tablet was in

900 ml medium, 4 paralell test were carried out in case of every compositions. The duration was 780 minutes, during that, 16 times 4 ml aliquot was taken and substituted immediately.

In the second test silymarin dissolution was investigated from Carbopol based hydrophilic matrix tablets in artificial gastric fluid. The medium contained 2.0 g NaCl and 80.0 ml of 1 M hydrochloric acid per liter, according to Ph. Eur. 9. The chemicals were obtained from Hungaropharma Zrt. Temperature of the medium was 37±0.5 ºC and it was constantly stirred through the tests, the paddle rotation speed was 100 rpm. Each tablet was in 900 ml medium, 4 paralell test were carried out in case of every compositions. The duration was 780 minutes, during that, 16 times 4 ml aliquot was taken and substituted immediately.

In the third test, complexated silymarin dissolution was investigated from Carbopol based hydrophilic matrix tablets in artificial intestinal fluid. Temperature of the medium was 37±0.5 ºC and it was constantly stirred through the tests, the paddle rotation speed was 100 rpm. Each tablet was in 900 ml medium, 4 paralell test were carried out in case of every compositions. The duration was 780 minutes, during that, 16 times 4 ml aliquot was taken and substituted immediately.

In the fourth test, complexated silymarin dissolution was investigated from Carbopol based hydrophilic matrix tablets in changed medium. For this test, the equipment's basket method was used. In the first hour of the test the tablets were in artificial gastric fluid, 4 ml aliquot was taken in every 15 minutes. Then the medium was changed to artificial intestinal fluid, and the test continued for 6 hours more, with 4 ml aliquot taken in every 30 minutes. Temperature of the medium was 37±0.5 ºC and it was constantly stirred through the tests, the rotation speed was 100 rpm. Each tablet was in 900 ml medium, 4 paralell test were carried out in case of every compositions.

Silymarin concentration of the samples were measured by spectrophotometric method at 287 nm (Shimadzu UV-1601 UV-VIS Spectrophotometer, Kyoto, Japan). For reference, 70 mg silymarin or silymarin-cyclodextrin complex containing 70 mg silymarin was solved in water then filtered. Sample absorbances are expressed as the percentage of the reference.

3. New scientific results

3.1. Particle size analysis

The average particle diameter of the first SEDDS formulation was 859 nm, and 65.5 nm of the second formulation. Based on the results of the particle size analysis, we can determine that the former is a self-microemulsifying drug delivery system (SMEDDS), while the later is a self-nanoemulsifying drug delivery system (SNEDDS), although the nomenclature is debatable.

3.2. Zeta potential analysis

The average zeta potential of the first composition was −71.3 mV, and −38.5 mV of the second composition. The magnitude of zeta potential in mV shows the stability of the system, 30 to 40 mV meaning moderate stability, 40 to 60 good stability, and over 61 excellent stability. The stability of the first composition was considered excellent, and that of the second composition was considered moderate, according to this categorization.

3.3. DPPH antioxidant assay

In the DPPH antioxidant assay the sample solutions were added to 0,06 mM reagent, and the absorbance of the color change was measured. The inhibition values were calculated using the formula:

$$I\% = \frac{A_0 - A_S}{A_0} \times 100$$

where A_0 is the absorbance of the 0.06 mM DPPH reagent, and A_s is the absorbance of the sample.

The compositions containig fenugreek extract had lower antioxidant capacity than the positive control ascorbic acid in lower concentrations, but on the same level in higher concentrations. The compositions containing only the SEDDS excipients without extract, had negligible antioxidant effect in any concentration.

3.4. Superoxide dismutase (SOD) antioxidant assay

The in vitro SOD enzyme activity assay was performed on Caco-2 cells. The SOD activity of the positive control cells treated with ascorbic acid dilution was taken as 100%. The SOD activity of the cells treated with SEDDS compositions containing fenugreek extract was higher than the SOD activity of cells treated with native fenugreek extract. The possible explanation could be that the cellular uptake was higher because of the penetration enhancing agents. The SEDDS compositions S1 and S2 also showed antioxidant effects that were mildly higher than those of the negative control PBS.

3.5. Permeability assay of fenugreek compounds

Permeability tests were carried out on cell monolayers presenting TEER values between 800 and 1000 Ω cm2. The complex metabolomical analysis carried out at the Department of Botany traced 13 different herbal compounds.

The compositions without fenugreek extract and the negative control PBS showed zero values for all compounds. Significantly more compound permeated from the SEDDS formulations, than from the native fenugreek extract. The SEDDS formulations also enhanced the absorption of soluble and well penetrating compunds, increasing their bioavailability. While the absorption of some compounds from the native extract does not show significant difference compared to the negative control, the penetration from the SEDDS systems always differs significantly from the native extract and the negative control. No significant difference was found between the two SEDDS compositions.

3.6. Cell monolayer integrity

The cell monolayer integrity was monitored using follow-up TEER measurements, the TEER values in certain points of time were recorded and expressed as the percentage of the starting TEER.

After the permeability assay, TEER values increased in the fresh medium, and after 24 h the values were above 90% of the baseline TEER. The samples added to the inserts loosened the tight junctions of the cell layer, but in the fresh medium, this was restored slowly. The TEER value after 24 h was close to the baseline, which points to the survival of the cells in the

fenugreek compositions. The reduction in TEER caused by Triton-X was constant until the end of the measurement, because the cells did not survive.

3.7. MTT cytotoxicity assay

After conducting the MTT assays, cell viability was expressed as percentage against the extract dilutions. The result of the blank PBS treatment was considered as 100% viability.

The Triton X-100 treatment used as positive control resulted in a 6.7% cell viability. According to the MTT test results, the Trigonella extract and the SEDDS compositions were also well tolerated by the Caco-2 cells, as all cell viability values were over 74%. A cell survival rate over 70% is required to consider a material non-toxic. In the case of the native extract, we experienced the highest cell viability values, while there was no significant difference between the cytotoxicity values of the different SEDDS compositions. No correspondence was found between the sample concentration and the cell viability.

3.8. Indirect dissolution test

During the indirect dissolution test, samples were taken at certain points of time from the medium. The samples were added to DPPH reagent, then the absorbance values were measured. The inhibition values were calculated using the formula:

$$I\% = \frac{A_0 - A_S}{A_0} \times 100$$

where A_0 is the absorbance of the 0.06 mM DPPH reagent, and A_s is the absorbance of the sample. The inhibition value of the medium is proportional to the dissoluted antioxidant herbal components. The antioxidant capacity of the fenugreek extract diluted in 100 ml distilled water as the positive control, and the antioxidant capacities of the SEDDS components (as negative control) were also displayed.

The dissolution was sustained and steady in the case of both the compositions and the capsule material. The hard gelatin capsules started to disintegrate after 30 min. The HPMC capsules achieved dissolution after 60 min. No significant difference was found between the dissolution profiles of the two SEDDS compositions.

3.9. Pharmaceutical tests

3.9.1. Weight uniformity

Weight uniformity test resulted in maximum deviation less than the allowed 5% (Ph. Eur. 9.). The highest deviation from the avarage weight was measured in the case of those tablets which contained Carbopol 71G, especially the composition without cyclodextrin. Tablets containing Carbopol 974 PNF showed the lowest deviation from the average, even as low as 0,5%.

3.9.2. Friability

In case of every tablet, friability loss was less than the allowed 1% (Ph. Eur. 9.). The highest friability loss occured in the case of tablets containig Carbopol 71G, while the compositions containing the other three Carbopols did not show significant difference in friability.

3.9.3. Hardness

As for the hardness test, the tablets proven to be well compressed. Compositions with Carbopol 71G proved to be the softest tablets, while compositions containing the other three types of Carbopol did not show significant difference in hardness.

Considering the results of the pharmaceutical test, we can state that the physical parameters proven to be proper. All our samples fullfilled the Ph. Eur. 9. requirements.

3.10. Carbopol cytotoxicity

Cytotoxicity of all the four Carbopols were tested by MTT assay on Caco-2 cell line. Correlation was not found between cell viability and Carbopol concentration. The highest cell viability occured in the case of Noveon.

3.11. Dissolution of silymarin from matrix tablets

In the first series of dissolution tests, the silymarin release from the Carbopol-based hydrophilic matrix tablets was examined. All the four Carbopols assured the sustained release of the silymarin. There was difference between the amount of released pharmacon, Carbopol

971P and Noveon being the two polymer with high amount of released silymarin after 780 minutes of dissolution. Carbopols were proven suitable as the base of a sustained release hydrophilic matrix tablet.

3.12. Dissolution of complexed silymarin from matrix

The second series of dissolution tests aimed to examine the pharmacon release in case of hydrophilic matrix technology combined with cyclodextrin complexation. The dissolution test for all the 16 combinations of the four types of β-cyclodextrin derivatives and the four Carbopols. After the comparison of the dissolution curves, two of the Carbopols (Noveon and Carbopol 974 P NF) were selected which shown the highest amount of released silymarin during the 780 minutes time period.

3.13. Dissolution of silymarin in artificial gastric fluid

In the third set of dissolution tests, the silymarin release of Carbopol based hydrophilic matrix tablets in artificial gastric fluid was examined. The pharmacon was complexed with hydroxy-propyl beta cyclodextrin. In this case all Carbopols shown the sustained drug release, but the dissolution was much slower in the process than in the previous tests with artificial intestinal fluid. In the end of the 780 minutes tests, the amount of dissolved silymarin was between 14% and 37%.

3.14. Dissolution of silymarin in altered medium

In the fourth set of dissolution tests, the medium was artificial gastric juice in the first hour, and then it was changed to artificial intestinal fluid for six hours more. The aim was to examine the behaviour of the Carbopol matrix in an environment with pH conditions similar to the human GI system. All the 16 Carbopol-cyclodextrin combinations were tested. In these cases, the amount of released silymarin was less than the previous tests, partly because of shorter time, partly because of the acidic medium in the first hour, which affected the dissolution. Comparing the dissolution curves, we could find those combinations of Carbopols and cyclodextrins which are supposed to have the best pharmacon release. These were Methylated beta-cyclodextrin combined with Carbopol 974 PNF and Noveon.

3.15. Comparison of the dissolution of silymarin from different formulations

Examining the results of the dissolution tests, two combinations of Carbopols and cyclodextrins assuring the most favourable properties for sustained release and increased bioavailability of silymarin can be selected. The dissolution test in altered medium was carried out for a commercial product, which was a conventional capsule with 70 mg silymarin as active ingredient. The dissolution curves of the commercial capsule and the Noveon and Carbopol 974 PNF based matrix tablets containig silymarin complexed with Methylated ß – cyclodextrin were compared. While the silymarin release of the conventional capsule peaked after the first hour, the matrix tablets implemented sustained release for six hours. The complexation with cyclodextrin increased the solubility of the active ingredient, resulting in a higher dissolved quantity. The dissolution curve of the commercial product differs significantly from the matrix tablets since the 15th minutes. The dissolution curves of the two matrix tablets did not differ significantly from each other until the end of the examination.

3.16. Cytotoxicity test of Noveon based matrix tablets

In the end, the dissolution test was repeated for the tablet containing the most suitable Carbopol-cyclodextrin combination, Noveon and methylated β cyclodextrin. The samples taken during the test were used for an MTT assay. Correlation was not found between the concentration of tablet components and the percentage of surviving cells. Cell viability was higher than 70% in case of each sample, and at certain points it was almost 100%. The assay proved the tablet cytocompatible (biocompatible).

4. Discussion

The increased interest toward oral formulations containing natural herbal active ingredients demands the incorporation into modern drug delivery systems, to make possible the effective and calculable therapeutic use of phytopharmacons with poor stability and bioavailability.

Fenugreek can be used for multiple therapeutic purposes, but primarily a potential agent in the therapy and prevention of diabetes and insulin resistence. In the first part of my PhD research two different SEDDS systems were formulated of standardised fenugreek seed extract.

Poor oral bioavailability is one of the main challenges in pharmaceutical sciences. And low aqueous solubility, low absorption rate and early degradation are the main causes of poor oral bioavailability. The SEDDS systems with suitable parameters can enhance the bioavailability of lipophilic, poorly permeable and unstable molecules.

Two different SEDDS systems were formulated of standardised fenugreek extract, one of the was in the micro-, and the other was in the nanometer range, both had proper stability, and increased the bioavailability of herbal compounds.

The antioxidant effects of the extract and the SEDDS formulations were examined by in vitro chemical and Caco-2 cell culture method. According to the DPPH reagent assay, the compositions containig fenugreek extract had lower antioxidant capacity than the positive control ascorbic acid in lower concentrations, but on the same level in higher concentrations. The SOD activity of the cells treated with SEDDS compositions containing fenugreek extract was higher than the SOD activity of cells treated with native fenugreek extract. The possible explanation could be that the cellular uptake was higher because of the penetration enhancing agents, and the antioxidant agents influenced the enzyme activity more.

During the permeability assay, the in vitro absorption of 13 different fenugreek compounds were examined on Caco-2 cells, 5 steroid saponins, 6 flavonoid glycosides, 4-OH-isoleucine and trigonelline. The permeability of the two SEDDS formulations was significantly higher in case of all 13 compounds than the native fenugreek extract, but they did not differ significantly from eachother. The membrane integrity after the permeability tests was monitored by TEER follow-up measurements.

Indirect dissolution test of the SEDDS formulations from hard gelatine and HPMC capsules was conducted. The hard gelatine capsule ensured sustained release for 30 minutes,

then disintegrated. The sustained release from the HPMC capsules lasted more than 60 minutes. The dissoluted active ingredients were measured indirectly by the use of DPPH reagent, based on the antioxidant effect of the fenugeek compounds. Several studies can be found describing the improved pharmacon release of SEDDS systems containig herbal compounds from different dosage forms.

Cytotoxicity was investigated by MTT method. Based on the results, the Caco-2 cells tolerated well the herbal extract and also the SEDDs formulations.

Silymarin has a wide range of effects, hepatoprotective properties principally. The main disadvantage of silymarin is its very low bioavailability, which affects the therapeutic effect. There are several possibilities to improve bioavailibility via solubility, permeability, metabolism, and excretion. In this study, different β-cyclodextrins were selected to increase the solubility profile of silymarin. Cyclodextrins are efficient excipients used in pharmaceutical industry for increasing solubility, bioavailability, stability and prevention of irritation and incompatibility. Complexation was performed by physical mixture method according to **Ghosh et al.**, who described the stability constant and phase solubility paramters of the complexes. The research was also based on the work of **Yeh et al**., who proven the solubility enhancement and increased efficiency of San Huang Shel Shin Tang containing multiple herbal compounds after complexation not only in vitro models, but in vivo experiments too.

Hydrophilic matrix technology was chosen to assure sustained release. It is a relatively cheap method, and the production does not require special equipment, devices for producing conventional tablets are suitable.

Carbopols are well-known materials used for topical gels, and their property of possessing carboxyl acid function makes their dissolution affected by the pH of the medium. The four types of Carbopol used as base for the matrix tablets were investigated by MTT cytotoxicity assay on Caco-2 cells. No correspondence was found between the Carbopol concentration and the cell viability. The highest cell survival rate was in case of Noveon, but none of the polymers found to be toxic.

20 different tablet compositions were prepared of the 4 polymers and the 4 polymers combined with the 4 complexing agents, by manual bench-top tablet press. The physical

paramters was verified with the weight uniformit, friability and hardness tests, according to the Ph. Eur. 9. All formulations fulfilled the requirements.

The silymarin release was sustained from all the 4 Carbopol-based hydrophilic matrix tablet in artificial intestinal fluid. Carbopol 971P and Noveon were the two polymer with the highest amount of released silymarin. Carbopols were proven suitable as the base of a sustained release hydrophilic matrix tablet.

The dissolution of silymarin complexed with different cyclodextrin derivatives from matrix tablets was sustained also, although significant differences occured between the 16 compositions in terms of released pharmacon. While the cyclodextrins resulted in similar dissolution profiles, from the Carbopols two could be selected (Noveon and Carbopol 974 PNF) as the highest amount of released silymarin during the 780 minutes test.

The dissolution of silymarin complexed with HPBCD was investigated in artificial gastric fluid, pH=1,2. The pharmacon release was sustained, but substantially slower than in artificial intestinal fluid. At the end of the 780 minutes test only 37% of the active ingredient was released at maximum. The decreased dissolution from the Carbopol matrix on lower pH is beneficial for the oral therapy, since the pharmacon liberation happens in the small intestines, not in the stomach.

The 16 different tablets containing silymarin-cyclodextrin complexes were also tested in altered medium. In the first hour of the experiment, the medium was artificial gastric juice, then which was changed to artificial intestinal fluid for 6 hours more, for the closer in vitro modelling of the pH conditions of the human gastrointestinal tract. Based upon the dissolution tests, two compositions were selected which are supposed to have the best pharmacon release: methylated beta-cyclodextrin combined with Carbopol 974 PNF and Noveon.

The dissolution profiles of the two selected formulation were compared to the dissolution profile of a commercially available conventional silymarin hard gelatine capsule. In case of the later, the silymarin release peaked after one hour, while the sustained release of the matrix tablets lasted 6 hours after the start of the experiment. The complexation with cyclodextrins increased the solubility of the pharmacon, and resulted in better dissolution.

The MTT assay carried out on the most proper composition showed high cell viability, proven the safe applicability of the tablet.

5. Summary

During my PhD studies, the focus of my research was the bioavailability enhancement of herbal drugs. My aim was to formulate modern drug delivery systems which are able to make better use of the beneficial effect of phytopharmacons than dried plant parts, cost-effective and simple to produce in proper quality, easy to administer orally, and not cytotoxic.

In the first part of my work SEDDS formulations were prepared of dried extract of fenugreek seed with the use of different emulsifiers. According to my examinations, stabile micro- or nanoemulsion systems can be formulated with a proper composition, which possess high antioxidant capacity and significantly increase the intestinal permeability of active herbal components compared to the native fenugreek extract. Furthermore, they easily can be filled into hard gelatine or HPMC capsules, therefore administration is simple, and they do not show cytotoxic properties. With this technology, a formulation with better oral bioavailability was achieved, and it can make the prevention and therapy of diabetes and insulin resistance with a natural herbal drug product containing complex phytopharmacons.

In the second part of my work Carbopol based hydrophilic matrix tablet were formulated of silymarin extracted from milk thistle. This technology was also combined with molecular encapsulation by cyclodextrins. According to my examinations, the tablets met the requirements of the European Pharmacopoea 9th Edition, and enabled sustained pharmacon dissolution on the pH of the human gastrointestinal system. The modified release and the improved solubility can lead to enhanced bioavailability. The tablets and the matrix forming polymers are safe and non-toxic, their administration is simple, and they have significant therapeutic benefits compared to the conventional dosage forms.

6. Publications related to the dissertation of the candidate

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Registry number: DEENK/389/2022.PL
Subject: PhD Publication List

Candidate: Dávid Zsolt Sinka
Doctoral School: Doctoral School of Pharmacy

List of publications related to the dissertation

1. **Sinka, D. Z.**, Doma, E., Szendi, N., Páll, J., Kósa, D., Pető, Á., Fehér, P., Ujhelyi, Z., Fenyvesi, F., Váradi, J., Vecsernyés, M., Szűcs, Z., Gonda, S., Cziáky, Z., Kiss-Szikszai, A., Vasas, G., Bácskay, I.: Formulation, Characterization and Permeability Studies of Fenugreek (Trigonella foenum-graecum) Containing Self-Emulsifying Drug Delivery System (SEDDS).
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DOI: http://dx.doi.org/10.1016/j.ddtec.2018.06.005

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