Recent data for phytochemical analysis of *Ononis spinosa* L. and preformulation of its active ingredients with cyclodextrins

PhD thesis

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1. Introduction and aims

Recent years therapeutic use of medicinal plants had been growing more and more due to development of research of their chemical analysis and clinical trials. There has been increasing attention and faith to more natural therapies or prevention as medicinal herbs due to their bigger safeness and favorable or less side effects. However towards effectiveness and safeness we have to compounds of natural sources as to synthetic drugs.

Re-evaluating a traditional medicinal herb, defining a new field of indication or describing an unclear mechanism of effect needs serious analytical, investigatory background, and the applied chemical and biological methods have to be suitable for these investigations.

Re-evaluating of *Ononis spinosa* is important, since it is a traditional medicinal herb, but its chemical and clinical analysis is still not complete. Its root contains triterpenoids and isoflavonoids that can be promising compounds for certain diseases (cardiovascular and some malignant diseases).

In recent decades supercritical fluid extraction; that works usually with fluid state inert gases, likely CO_2 has been getting widespread method for obtaining mostly apolar molecules.

Apolar compounds of *Ononis spinosa* L. are the therapeutically useful phytosterols that are promising for treatment or prevention of benignus prostata hypertrophia (BPH) or some malignant diseases. Qualitative and quantitative analysis of sterols are performed typically by gas chromatography with derivatisation.

Utilization of cyclodextrins has been increased so far; there are more and more medicine encapsulated by CDs on the market. In chemistry of medicinal herbs number of compounds preformulated as an inclusion complex is still increasing; for example, flavonoids, catechins, essential oil or steroid components in the literature.

In practice minimizing of bitterness in different juices by eliminating naringin, hesperidin, and limonin; eliminating caffein from coffee and tea; eliminating cholesterol from butter, egg and other dairy products have been succeeded.

In addition, cyclodextrins may influence effect of medicinal compounds in the human organism, they can increase biological availability; e.g. enhancing antioxidant effect of polyphenols, flavonoids.

Cyclodextrins can contribute to improved bioavailibility of phytomedicines with decreased doses and therefore better side effect profile.

In our work we aimed at investigating and identificating compounds of *Ononis spinosa* L. root. Firstly, we aimed to qualitative and quantitative evaluate triterpenoids in different extracts of the root obtained by different organic solvents. We also aimed to study biological availibility of complexed forms of genistein and daidzein with different cyclodextrins; and studied effect of cyclodextrins on isoflavonoid extractions. Aims are listed below in points:

1. Extractions of triterpenoids of Ononidis radix with different organic solvents and supercritical fluid CO_2;. qualitative and quantitative evaluation of triterpenoids in each extract by GC-MS without derivatisation.
2. Determination of β-sitosterol content in the different extractions by GC-FID without derivatisation.
3. Identification of isoflavonoids in *Ononis spinosa* root by HPLC-MS.
4. Inclusion complex forming of genistein and daidzein with β-, γ-, hydroxy-propyl-β- and random-methyl- β-cyclodextrin; investigation of complexes by CD-spectroscopy and ^{1}H-NMR spectroscopy.
5. Investigating of dissolution profiles and membrane transport of the prepared complexes across Caco-2 cell line.
6. Investigation of effect of genistein and genistein/β-cyclodextrin on lifespan of *Caenorhabditis elegans* modell organisation.
7. Extraction of isoflavonoids by means of different cyclodextrins; studying extraction conditions.

2. Materials and methods

2.1. Materials

For the triterpenoid analysis, *Ononis spinosa* L. (Fabaceae) root originated from the Biohorticulture Bio-Berta (Kiskőrös, Hungary); for the isoflavonoid analysis, the plant material was purchased from the Rózsahegyi Medical and Spice herb Ltd (Erdőkertes, Hungary) and in both cases was dried, chopped and ground. This powdered plant material was used for the extractions. The analytical standards of β-sitosterol, β-amyrin, stigmasterol, campesterol and 5α-cholestan-3-on were purchased from Sigma-Aldrich (St Louis, MO, USA), genistein and daidzein substance of analytical purity were purchased from LC Laboratories (Massachussetts, USA). Cyclodextrins: β-CD, γ-CD, hydroxypropyl-beta-cyclodextrin (HP-β-CD) and randomly methylated beta-cyclodextrin (RAMEB-CD) were of pharmaceutical grade materials, manufactured by Wacker Chemie, Germany. All other chemicals used throughout the study were of analytical grade (petroleum spirit and KOH from Reanal, Budapest, Hungary; chloroform, hexane, 96% ethanol and ethyl acetate from Molar Chemicals, Budapest. Hungary; toluene from Carlo Erba, Milan, Italy). Methanol used for HPLC measurements were analytical grade from Reanal Kft (Budapest, Hungary; marked Carlo Erba). Degree of substitution of HP-β-CD was 4.2 and that of RAMEB-CD was ~12.

2.2. Investigations of triterpenoids

2.2.1. Extraction

2.2.1.1. Soxhlet Extraction

Soxhlet extractor, normal way at ambient pressure, at the boiling point of the solvent; n-hexane until the solvent became colourless; after extracting again, with 96 % ethanol; isopropanol, ethyl acetate, in the same way as with n-hexane.

2.2.1.2. Solvent extraction under mild conditions

Laboratory setup: spheric flask, bladed stirrer at 40 °C. Solvents: acetone, ethanol and methanol. Extraction: 3x1 h; extracts were collected, solvent evaporated in a Büchi Rotavapor R-200 under vacuum.

2.2.1.3. Supercritical fluid extractions

- **Laboratory scale supercritical fluid extraction**

ISCO 2-10 experimental laboratory supercritical extractor (ISCO, USA) with CO_2; at 40 °C; on 100-200 bar; extraction time: 60 and 90 min for each pressure; first 30 min: soaking in liquid CO_2 without pressure (static extraction), further 30 or 60 min: pressure set to the prescribed value (dynamic extraction); absorbing of extracts in 96 % ethanol. These extracts are numbered SFE 1–4.

- **Pilot scale supercritical fluid extraction**

Semi-industrial supercritical extractor; liquid CO_2; at 40 °C; on 450 bar; total extraction.

2.2.2. Sample preparation

Extracts concentrated under vacuum; saponifying (to cleane them from oily materials), 96 % ethanol and KOH solution, hot water-bath; extracts diluted with water; shaken with petroleum ether; organic phases collected; washed with water until neutral; evaporated in a Büchi Rotavapor R-200 under vacuum. Dry extract weighed; dissolved in chloroform. These unsaponified extracts were then analysed. In these extracts the sterols and triterpenes were present in free forms.

2.2.3. Analysis

2.2.2.1. Gas chromatography

- **GC-MS conditions for analysis of triterpenoids**

Agilent 6890N gas chromatograph coupled to a 5973N quadrupol mass analyzer. HP-5MS (30 m x 0.25 mm ID x 0.25 μm) column (Agilent, USA); oven temperature: 140°C for 1 min after injection then programmed at 10°C min^{-1} from 140 to 270°C, (20 min isothermal) then from 270 to 300°C at 10 °C min^{-1} (6 min isothermal); carrier gas: helium at 1 mL min^{-1}; injector temperature: 280°C; injected volume: 1 μL. The identification of the compounds was done by comparing the retention times, Kovats indices and mass spectra with spectral data of

authentic standards and the NIST 05 spectral library; the percentage ratio of the different triterpenoids were determined by area normalization.

- **GC-FID conditions for β-sitosterol contain**

Agilent 6890N instrument; DB-5MS (25 m x 0.2 mmID x 0.33 μm) column (Agilent, USA); carrier gas: Helium; flow rate: 1 mL min^{-1}; injector temperature: 280°C; injected volume: 1 μL; oven temperature: 120°C for 1 min, then from 120 to 300°C at 10°C min^{-1} (14 min isothermal), then from 300 to 310°C at 10°C min^{-1} (10 min isothermal); temperature of the flame ionisation: was 330°C; internal standard: 5α-cholestan-3-on (quantitative determination). Identifications were based on standard addition and retention data. Concentration range of β-sitosterol: 1–100 μgmL^{-1} at six different points; concentration of the internal standard: 12.65 μgmL^{-1}. Blank: chloroform was analysed in order to confirm that the solvent did not contain any impurities at the same retention time as the compounds of interest.

2.3. Investigations of isoflavonoids

2.3.1. Identification of isoflavonoids in Ononidis spinosae radix by HPLC-MS/MS

Agilent 6410 Triple Quad LC/MS system (Wilmington, DE, USA); electrospray ionization; positive ion mode; Zorbax Eclipse XDB-C18 reversed-phase column (150 × 2.1 mm i.d.; 5 μm particle size, Agilent, Santa Clara, CA, USA); solvent: 0.3% acetic acid (A) and methanol (B); gradient elution: 0.00 min, 29% B; 20.00 min, 85% B; 22.00 min, 100% B; 27.00 min, 100% B; 32 min, 29% B; flow rate: 0.25 ml/min; column temperature: 25°C; injection volume: 2 μl. The experimental conditions for MS: nebulizer: 45 psi; drying gas flow rate, 9 $Lmin^{-1}$; drying gas temperature, 350 °C; capillary voltage, 3500 V; fragmentor voltage 100 V; MS scan from m/z 150 to 1200; collision energy, 12, 20, 28 and 36 eV.

2.3.2. Preparing inclusion complex of isoflavones with β-, γ-, HPB- és RAMEB-cyclodextrins

2.3.2.1. Computer modeling studies for visualization of host–guest fitting for genistein and daidzein with β- and γ-CD

All calculations were run on Spartan for Windows ‘06 software version 1.1.1. The input structure of ligand (genistein) was taken from the database of SPARTAN. In case of both CDs, explicit watermolecules were removed and missing hydrogen atoms were added. CD and ligand structures were pre-optimized separately using the PM3 semi-empirical method. A centroid was defined for both CDs as the geometrical center of alpha-carbonic atoms their sugar units. The structures of input complexes were generated by the superposition and manual alignment of genistein’s 4H-pyran-4-on ring to these centroids. Cyclodextrins and the inserted genistein were optimized together using the PM3 semi-empirical method. Optimizations were completed successfully, the calculations were converged, and there was no sign of major overlapping between Van der Waals surfaces of the CDs and the ligand in the output complexes.

2.3.2.2. Phase-solubility studies

Registered in deionized water; 25ºC after a 10-h equilibration time; by equilibrating excess amounts of solid, crystalline genistein and daidzein in aqueous cyclodextrin solutions that contained increasing CD concentrations in away, that always excess, undissolved solid genistein and daidzein remained in the solubility test. Stirring for 10 h at 25ºC the undissolved genistein and daidzein; filtered off on a membrane filter of 0.45 μm; assayed for dissolved genistein and daidzein concentration by UV-spectrophotometry. It was also proved that the presence of β-CD, γ-CD, HP-β-CD and RAMEB-CD had no effect on the spectrophotometric determination of genistein in water.

2.3.2.3. Preparation of cyclodextrin complexes

Complexes of genistein and daidzein with β-CD, γ-CD, HP-β-CD and RAMEB-CD were prepared by wet-kneading method in ceramic mortars. Calculated amounts of genistein /

daidzein and the CDs, homogenized; wetted with deionized water; kneaded intensively for 30 min yielding a nearly solid dense dough; drying at room temperature in air, to constant weight; complexes ground to fine powder and sieved to particle size of about 160 μm. The water content of the binary complexes was determined by loss on drying method; at 105°C in vacuum, to constant weight in an open container over phosphorous-pentoxide.

2.3.2.4. Dissolution profile of genistein and daidzein and their inclusion complexes

Under non-sink conditions; 37°C, certain amount of complexes taken into deionized water; samples withdrawn from the dissolution medium at different time points (at 5, 10, 15, 20, 25, 30, 35, 40, 50, 60 minutes) released genistein and daidzein concentration determined by UV-spectrophotometry.

2.3.2.5. CD spectroscopy of prepared complexes

The spectra were recorded using Jasco J-815 spectropolarimeter using a 10 mm cylindrical quartz cell. The spectra were recorded in 200-350 and 200-400 nm wavelength range. The spectrawere accumulated five times with a bandwidth of 0.1 nm a scanning step of 0.2 nm at a scan speed of 20 $nm.min^{-1}$. The solvent used for recording was distilled water. The complexes were sieved, dissolved in distilled water and was centrifugated than the filtrate was measured.

2.3.2.6. ^{1}H-NMR investigations of the prepared complexes

^{1}H-NMR measurements were carried out on a Varian Inova spectrometer (600 MHz for ^{1}H) equipped with a dual 5-mm inverse-detection gradient (IDPFG) probehead. All spectra were recorded at 30.0±0.1°C and referenced to the residual HDO signal (4.72 ppm). The kneaeded solid sample was dissolved in D_2O and shaked at room temperature for 48 h to achieve the equilibrium. The obtained suspension was centrifuged and supernatant saturated solution was introduced into the NMR tube.

2.3.3. Investigation of isoflavones and their CD complexes in biological assays

2.3.3.1. Investigation membrane permeability of genistein and daidzein and their inclusion complexes in Caco-2 cell line

Cells were maintained by regular passage in Dulbecco's modified Eagle's medium (Sigma–Aldrich), with 10% heat-inactivated foetal bovine serum (Sigma–Aldrich), 1 % nonessential amino acid (Sigma–Aldrich), and 100 mg L^{-1} gentamycin; 37°C in an incubator containing 5% CO_2. The passage number of the cells was between 25 and 42. Caco-2 cells were seeded at density of 200,000 cells/well on Transwell® (Corning Costar, Lowell, MA, USA) polycarbonate filters (pore size 0.4 :m, surface area 1.12 cm^2). Culture medium replaced with fresh medium every 2 days; TEER was measured with a Millicell–ERS voltohmmeter (Millipore, Budapest, Hungary); inserts were used at TEER values between 1500 and 2000 Ω × cm^2. In transport experiments, TEER values were also measured at the end of sampling to check monolayer integrity.

Quantitation of the genistein and daidzein: samples from basolateral side, by a HPLC-DAD-UV system (Jasco). injection vlume: 20 µl; SUPELCOSIL LC-18-DB column (250 x 4.6 mm, i.d. 5 µm); flow rate: 0.5 ml/min; mobile phases: solvent A: 0,3 % acetic acid, solvent B: 100% methanol; gradient: from 38 to 100% solvent B over 7 min, isocratic at 100% solvent B over 4 min, and isocratic at 38% solvent B for 5 min.

2.3.3.2. Effect of genistein and genistein/ β-CD on lifespan of *Caneorhabditis elegans*

Isolation of larval stage ('synchronisation'); L4 larvals to NGM (nematode growth medium) agar medium with FUDR (5-fluoro-2'-deoxyuridine)) antimetabolite in it; 500 mg complex and equvalent amount of genistein into 50 ml NGM solution; sonication; mixing; taken to NGM containing medium; after 9th day animals to new medium free of drugs and FUDR; deaths registered. Results from average of 50-50 individuals, then lifespan curves taken.

2.3.4. Extraction of isoflavonoids by means of cyclodextrins comparing traditional extracts

1.5 g ground and sieved plant material; solvent: 30% ethanol / distilled water; extraction time: 75 min by sonication; solutions added 0 M (control extraction), 0,01 M, 0,03 M and 0,05 M of β-, γ-, HP-β- and RAMEB-CD; centrifuged on 7000 rpm; filtered on 0,22 μm syringe filter, than analysed. DS of HP-β-CD was 4.2 and that of RAMEB-CD was ~12.

3. Results and discussion

3.1. Results of investigation of triterpenoids

3.1.1. Identification of triterpenoids in Ononis spinosa root by GC-MS without derivatisation

Based on GC-MS investigations without derivatization of unsaponifiable extracts of spiny restharrow root, sterol compounds β-sitosterol, stigmasterol, campesterol, stigmastan-3,5-diene and the triterpene derivatives β-amyrin and α-onocerin were identified. The Kovats indices of the identified compounds were 3304; 3230; 3260; 3342 and 3318 for stigmastan-3,5-diene, campesterol, stigmasterol, β-sitosterol and β-amyrin, respectively, and showed good agreement with literature data. A similar situation applied to the retention times (22.85; 26.35; 27.37; 29.40; 30.51 min for stigmastan-3,5-diene, campesterol, stigmasterol, β-sitosterol and β-amyrin). We found also an unknown component of retention time: 37.16 min in the TIC chromatogram, which had the same molecular mass as α-onocerin (442). Since pure α-onocerin can be obtained from the dried plant material by microsublimation, this compound was isolated by us and its identity confirmed by TLC. We found good agreement between the retention time and MS spectra of α-onocerin and the unknown component of extracts, confirming that the constituent in question was α-onocerin and its calculated Kovats index was 3521 (there are no published values to compare it to).

3.1.2. Investigations of triterpenoid and β-sitosterol content influenced by different extraction methods

It has been found that the relatively highest amount of nonsaponifiable material was obtained by the supercritical fluid extractions and hexane extraction, followed by isopropanol and ethylacetate extraction. Among ths supercritical fluid extracts those made on 100 bar were proved more nonsapnofiable fractions. Hexane and SFE extracts contained relatively the largest quantity of β-sitosterol; however, the alcoholic extracts has still significant amounts of triterpenoids and β-sitosterol. These results imply that β-sitosterol exists partially in a chemically bound form in spiny restharrow root. Studying the influence of changing parameters of the laboratory supercritical fluid extraction methods on β-sitosterol content it was shown that the extracts 100 bar was the better condition for extracting triterpenoids; No.1 contained the highest level the extracts No. 3 and 4 had almost the same level of β-sitosterol,

extract No.4 had a surprisingly lower level than that of the hexane extract. Beside quantifying the β-sitosterol content in the saponified fractions from the various extracts, we also studied the triterpenoid composition in the extracts obtained by traditional solvent extractions. It was found that the percentage of β-sitosterol was the highest in hexane and pilot scale SFE extracts followed by the ethanolic and methanolic extracts. The methanolic and the pilot scale SFE extracts also contained campesterol, in contrast to the other extracts as they contained no campesterol whatsoever. The highest percentage of α-onocerin as found in the ethyl acetate extract which can be explained by the specific triterpene structure of α-onocerin. Stigmasterol was not detected in the hexane, the ethyl acetate or the isopropanol extracts, the same was true for β-amyrin. Stigmastan-3,5-diene were determined in every fraction. We confirmed that the pilot scale SFE extract was the most selective for triterpenoids (93.3%) followed by the hexane extract (71.8%). The relatively high percentage of triterpenoids in the ethanol and methanol extracts was probably caused by the fact that triterpenoids are partially in bound form in the dried plant material and the more polar solvents are more suitable to extract them. Although the total yield of the extraction by SFE is lower than that observed with traditional methods, it is much more selective for triterpenoids.

3.2. Investigations of isoflavones

3.2.1. Identification of isoflavonoids in Ononidis root.

Compounds yielded from aqueous and 30% ethanolic extractions were determinated by HPLC-MS/MS. The ESI-MS spectrum of the 30% aqueous ethanolic extract proved several molecule ion, which take place in range m/z 400-600. The product ions were analysed by means of literature data. Finally, components in question were determined based on their molecule ions, retention times, UV maximum and product ions; comparing to previously reported data.

The isoflavonoids extracted from Ononidis radix with 30% ethanol were mostly glycoside/galactoside or malonyl-glycoside. We gained the following molecule ions $[M+H^+]$: *m/z* 433, 431, 447, 477, 517, 519, 533, 563. The molecule ion at m/z 433 may mean genistein-7-O-β-D-glycoside (genistein) with the 269 production as its aglycon Three molecule ions at *m/z* 447 signed two calychosin-glycoside and the last one is maackiain-glycoside. Molecule ion at m/z 431 with an m/z 269 product ion may be characteristic for

formononetin-7-O-β-D-glycoside (ononin) and its aglycon, respectively. Two molecule ions at *m/z* 517 molecule ions are tentatively two formononetin-glycoside isomer or glicoside/galactoside „twins". Two other molcules at *m/z* 519 are genistein-7-O-glycoside-malonate, and medicarpin-7-O-glycoside-malonate. We found two new isoflavonoid; the irisolidone-glycoside (*m/z* 477) and calychosine-glycosid (*m/z* 447), and their malonate derivatives (*m/z* 563 és 533).

3.2.2. Investigation of inclusion complexes of genistein and daidzein

3.2.2.1. PM3 optimization for matching of genistein and daidzein with cyclodextrins

Optimizing the molecular fitting of genistein/β-CD and genistein/γ-CD spatially with semi-empirical PM3 method, it can be seen that geometrical matching of the two molecules is really possible. On the other hand, it was also indicated by the computer modeling study that α-CD will hardly form a well-fitted host–guest type complex with genistein.

3.2.2.2. Phase-solubility study

Phase solubility curves of genistein and daidzein showed increasing tendency with all of CDs in the 0-10% concentration range. The best enhancment could be obtained for the substituted CDs; HP-β- és a RAMEB-CD.

3.2.2.3. Dissolution profile

In the dissolution profiles it is can be well oberved that the parent cyclodextrins have lower influence to solubility of the genistein and daidzein, and the substituted cyclodextrin derivatives HP-β-CD and RAMEB-CD resulted in really high solubility enhencement for the two isoflavones. Although both γ-CD complexes showed a relatively higher peak of concentration at the beginnig, it happened probably because of the better wettability. Basically, the native cyclodextrins have a limited solubility because in higher concentrations they can form aggregates, especially the beta-cyclodextrin through hydrogen bonds, while in derivates the aggregate forming are practically „blocked" or limited by the substitutes.

3.2.2.4. Results of ^{1}H-NMR investigation of genistein and daidzein / CD complexes

The sufficient signal to noise ratio for a typical 2D ROESY experiments was only achieved in the genistein/β-CD system. The expansion of the spectrum contains intramolecular cross-peaks between the aromatic dubletts (7.06ppm and 7.39 ppm) of the 4-hydroxyphenyl moiety. The protons at 7.39 ppm show a weak cross-peak between the singulet of the ring B (7.96 ppm) indicating that the protons at 7.39 ppm are in the meta-position the others at 7.09 ppm are in the ortho-position, respectively. From the viewpoint of encapsulation, intermolecular cross-peaks of the 4-hydroxyphenyl dublets with the inner CD protons H-5 (at 3.88 ppm) and H-3 (at 3.97 ppm) are important. The relative cross-peak intensities revealed that the whole aromatic ring is in the β-CD cavity and the mode of penetration occurred from the wider rim side. A further evidence for this type of orientation of genistein is a weak cross-peak between the ring B singulet (7.96 ppm) and the H-5 proton of β-CD (3.88 ppm).

3.2.2.5. Results of CD spectroscopy of genistein and daidzein / cyclodextrin complexes

Complex formation is confirmed for all of the complexes by CD spectroscopy. Genistein and daidzein as achiral molecules do not have a CDi spectrum, buti in chiral sorrounding becomes chirally perturbated and produce an induced CD spectrum (ICD). At genistein/ γ-CD genistein has probably a different orientation than at β-CD derivatives. The β-CD and HP-β-CD resulted in a negative band, but its form is different, therefore it can be supposed that the two complexes do not have the same structure. The ICD spectrum at γ-CD is a positive curve that implies that genistein has different orientation to the CD molecule. As for RAMEB-CD, its spectrum is different from the other spectrum probably because of the diffusion of light caused by particles of colloidal size.

At CDi spectra of daidzein/ β-CD an intensive positive band could be observed; similarly to daidzein/ HP-β-CD, but here is a negative banda t 350 nm that may be caused by a different structure. The spectrum of daidzein/ γ-CD contrary to the complexes of β-CD derivatives implies a very different structure of the complex. In case of daidzein/ RAMEB-CD the CDi sign is the biggest one, and its structure is similar to that of β-CD.

3.2.3. Investigation of isoflavones and their CD complexes in biological assays

3.2.3.1. Investigation of membrane permeability of genistein and daidzein and their CD complexes in Caco-2 test

In preparing test solution we utilized the highest solubility of genistein and daidzein for each CDs to observ the kinetics of the transport. The apparent permeability coefficients of genistein and its CD complexes imply that encapsulated genistein is transported through the monolayer faster than pure genistein. The RAMEB-CD hardly improved the kinetics, it may be caused by more stable complex forming than that of the other CDs nonetheless showed the best effect on dissolution of genistein. The γ-CD proved the best enhancing of transport kinetics, followed by HP-βCD and β-CD.

Since test solutions had different concentrations of the isoflavones, based on the maximum solubility, different amounts of genistein were transported across the monolayer. In contrast, pure genistein could cross the cell line with one order of magnitude. Although RAMEB-CD enhanced solubility of genistein the best, not the genistein/RAMEB-CD complex showed the highest transport kinetics but the genistein/γ-CD.

On the whole, comparing the concentrations of the test solutions, the transport kinetics and the transported genistein, the cyclodextrins could enhanced not only the solubility but also the transport across the Caco-2 monolayer, but not equally.

As for kinetics of daidzein and its CD complexes, we prepared the test solution the same way as for genistein; according to the highest solubility of the daidzein/CD complex.

The values of calculated apparent permeability coefficient implies that both genistein and daidzein can pass easily through the monolayer of the caco-2 cells as soon as they are dissolved in the aqeous system. Since test solutions had different concentrations of daidzein (according to value of the highest solubility) so amounts of transported daidzein were different. Accordingly, the more daidzein of the test solution had the more amount of daidzein was transported across the monolayer. The kinetics of transport had the same order of magnitude in the presence of CDs, so tentatively CDs increased the permeability by enhancing of solubility.

3.2.3.2. Effect of genistein and genistein/β-CD complex on lifespan of *Caenorhabditis elegans*

The results were 95 and 96% of the individuals were alive counted on 10th day on genistein containing medium, incase of genistein/β-CD this number was 97-100%; on „empty" (free of drug) medium it was 86%. On 14th day the different is much bigger: the „empty" on medium 30% of the animals were alive; at the β-CD containing medium they were 43%, at genistein and genistein/β-CD it was 61-65 and 86-89%, respectively. On 15th day the difference was even bigger: 22, 39, 51-54 and 82-87% for empty, β-CD containing, genistein and genistein/β-CD containing medium. Its clearly seen, that complex forming extremely enhanced bioavailability of genistein.

3.2.4. Evaulating of isoflavonoid content in extracts of Ononidis radix

3.2.4.1. Effect of CD derivatives on extraction of isoflavonoids in Ononidis radix

- **Partition of isoflavonoids in 30%-os ethanolic extract performed with cyclodextrins**

The HP-β-CD extracts have the greatest isoflavonoid yield and the γ-CD extracts have the smallest one and the differences between them inversely proportional with the increasing concentrate of CDs. Although studying control extraction it seems to be more efficient even than γ-CD extractions caused probably by having high aggregative property of γ-CD.

During the extraction process CDs assisted isoflavonoid yield at the following order: HP-β-CD > β-CD > RAMEB-CD > γ-CD from the *Ononis spinosa* root. However, the yield of the control extract was even higher, only that of at HP-β-CD was significant amount; although similar yield to that of the control extract.

Isoflavonoids has been obtained in different amount depending on cavity size, polarity, substitutes and side chains. RAMEB-CD had less effectiveness than in aqueous extracts, and likely not for the malonil derivatives. γ-CD and HP-β-CD extracted even malonyl derivatives from Ononis root. Genistin could be extracted in biggest amount, as a main component.

These results all confirmed that ethanol despite of extracting isoflavonoids and usually flavonoids very efficiently, in these experiment practically suppress effect of CDs in extraction of isoflavonoids; since ethanol molecules themselves are in competition for CD

cavity. Thus, ethanol has stronger features opposite to CDs. Effect of β-CD and HP-β-CD relatively surpass the control but yields are inversely proportional to concentration grade.

- **Partition of isoflavonoids in aqueous extract performed with cyclodextrins**

In aqueous extracts the effect of cyclodextrins can be found specifically; almost all of the extracts surpassed the control extract. The greatest yields were obtained at 0,01 M CD concentration with significant increasing, however concentration grade was inversely propotional to isoflavonoid yields.

CDs enhanced the extracted isoflavonoid amounts in the following order: γ-CD < RAMEB-CD < HP-β-CD. The γ-CD was the less effective, probably due to its high aggregation forming property. Its efectiveness was the highest at 0,01 M and little bit lower at 0,03 and 0,05 M, however d not reach the isoflavonoid amount of the control extract. Lower efficacy of RAMEB-CD than HP-β-CD can be caused by smaller affinity of more polar isoflavonoid-glycoside to the enter CD cavity substituted by apolar methyl groups.

Ratio of some minor componenst slightly increased with increasing CD concentration, like formononetin-glycoside, maackiain-glycoside-malonate, other components have decreasing amount, for example genistin, a major isoflavonoid, which is present in RAMEB-CD and HP-β-CD extracts on highest amount. Beside genistin, calichosin-7-O- glucoside and malonate of calichosin-7-O- glucoside and ononin are present in RAMEB-CD extract by decreasing amount. The two malonates of formononetin-7-O-monohexoside and irisolidon-7-O-glucoside occurs likely in HP-β-CD and RAMEB-CD extracts and their amounts do not increase with CD concentrations. The irisolidone-glycoside-maolnate is especially in the γ-CD and HP-β-CD containing extracts. The malonyl derivative of medicarpin-7-O-glucoside is present in HP-β-CD extract in higher amount.

4. Publications:

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2. Daruházi Á, Szente L, Balogh B, Mátyus P, Béni Sz, Takács M, Gergely G, Horváth P, Szőke É, Lemberkovics É. (2008) Utility of cyclodextrins in the formulation of genistein: Part 1. Preparation and physicochemical properties of genistein complexes with native cyclodextrins. J Pharm Biomed Anal 48(3): 636-640.

3. Daruházi Á, Szarka Sz, Héthelyi É, Simándi B, Gyurján I, László M, Szőke É, Lemberkovics É. (2009) Terpenoidok az *Ononidis spinosae radix* szuperkritikus és hagyományos kivonataiban. Olaj, szappan, kozmetika 58: 25-31.

4. Daruhazi A. (2011) Cyclodextrins for flavonoid formulations. Cyclodextrin news 25(4): 1-10.

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