INTRODUCTION

The lipophilic extract from seeds of Silybum marianum, a milk thistle plant is used widely as a hepatoprotective herbal drug including United States, Asia and Europe (Wellington et al. 2001, Dehmlow et al. 1996). It is a mixture of several different flavonolignans and flavonoids. The active complex is composed of four isomer flavonolignans (silybin, silydianin, isosilybin, and silychristin) collectively known as silymarin. Silybin (Silybin A and Silybin B) is the most biologically active component and makes up 50% to 70% of silymarin. Silybin is followed by silychristin (20%), silydianin (10%), and isosilybin (5%) (Saller et al. 2001.) (Pradan et Girish 2005.)


The therapeutic effects of silymarin are restricted due to its low bioavailability after oral administration (Blumenthal et al. 2000, Wachter et Zaeske 2000, Voinovich et al 2009). Only 20-50 % of orally administered Silymarin can be absorbed from the gastrointestinal tract, where it undergoes extensive enterohepatic circulation (Wu et al.2009). The four major causes of limited silymarin bioavailability are extensive phase II metabolism, low permeability across intestinal epithelial cells, low aqueous solubility, and rapid excretion in bile and urine (Shamama et al 2011).

A number of formulations have been used to increase its solubility and bioavailability: incorporation in solid dispersion (Chen et al., 2005), solid lipid nanoparticles (He et al., 2007) and formulation of self emulsifying drug delivery system (Woo et al., 2007, Wei et al., 2006), complexation with phospholipids (Yanyu et al., 2006).

Arcari et al. increased the solubility of silymarin by complexation with β-cyclodextrin. Albeit the good solubility data, the oral administration of β-cyclodextrin is limited because of its cytotoxic property. Our previous experiments we found that new generation β-cyclodextrins have got low cytotoxic effect because of the chemical modifications, nevertheless they can increase the solubility thereby the bioavailability of insoluble drugs (Kiss et al.2010.) (Fenyvesi et al 2011.).

In this study our aims were to form cyclodextrin-silymarin complexes with new generated β-cyclodextrins with low cytotoxicity: (heptakis(2,6-di-O-methyl)-β-cyclodextrin (DIMEB); randomly methylated-β-cyclodextrin (RAMEB); hydroxypropylated-β-cyclodextrin (HPBCD); quaternary amino-β-cyclodextrin polymer (QABCDP). We also planned to confirm the existence of the CD-silymarin complexes and examine their solubilisation properties.

MATERIALS AND METHODS

Silymarin was extracted from the seeds of Silybum Marianum with a validated extraction method and fully characterized at the Department of Applied Chemistry, University of Debrecen. The silibinin (silybin A and B) content was 31 m/m%. Hydroxypropyl-β-cyclodextrin (HPBCD), randomly methylated β-cyclodextrin (RAMEB), 2,6-di-O-methyl β-cyclodextrin (DIMEB) and Quaternary amino β-cyclodextrin polymer (QABCDP) were purchased from Cyclolab Ltd. (Budapest, Hungary).
All other chemicals were of analytical grade and obtained from Sigma-Aldrich (Hungary).

**Preparation of silymarin-cyclodextrin complexes**

Silymarin-cyclodextrin complexes were produced with HPBCD, RAMEB, DIMEB and QABCDP. Complexes were prepared by kneading method. Briefly, 1.00 g Silymarin was dissolved in ethanol 96% and mixed with 20.00 g cyclodextrin in a mortar, than the mixture was dried at 30 °C. After drying the products were mixed thoroughly again. By this process silymarin-cyclodextrin complexes with 1:20 mass ratio were obtained.

**Mass spectrometric analysis**

Silymarin-HPBCD complex was dissolved in distilled water and mass spectrometric analysis was performed as follows. Electrospray Quadrupole Time-of-Flight MS (ESI-QqTOF), measurements were performed with a MicroTOF-Q type QqTOF MS instrument equipped with an ESI source from Bruker (Bruker Daltoniks, Bremen, Germany). The sample solutions were introduced directly into the ESI source with a syringe pump (Cole-Parmer Ins. Co.) at a flow rate of 3 µL/min. The temperature of the drying gas (N₂) was maintained at 180 °C. The voltages applied on the ESI source were 4 kV. All the MS spectra were accumulated and recorded by a digitizer at a sampling rate of 2 GHz. Each spectrum was calibrated externally with the salt of sodium trifluoroacetate. The mass spectra recorded were evaluated by the DataAnalysis 3.4 software from Bruker.

**High performance liquid chromatography**

Silymarin-cyclodextrin complexes were dissolved in distilled water at the concentrations of 0.5, 1, 2, 10 mg/ml. All of the solutions were filtered through a membrane filter with 0.22 µm pore size and the solutions were analyzed quantitatively by HPLC. The HPLC system consisted of Waters 2695 separation module, Waters Symmetry C18 column, the column temperature was 40 °C. We used Waters 2996 Photodiode array detector and the detection wavelength was 288 nm. The eluent contained 0.1% formic acid in water and methanol. For quantitation silibinin standard (silybin A and silybin B) was used.

**RESULTS AND DISCUSSIONS**

**Identification of silymarin-HPBCD complex**

In order to identify the silymarin-cyclodextrin complexes and confirm their existence in water we performed mass spectrometric analysis. It is expected that in the case of strong interaction between silymarin and cyclodextrin the complex is detectable and appears on the mass spectra. We selected silymarin-HPBCD complex for testing our hypothesis as HPBCD efficiently solubilise lipophilic molecules in water (Cserháti et al., 1998). Fig. 1. shows a typical mass spectra obtained by the analysis of 1:20 (m/m) silymarin-HPBCD complex. On the spectra protonated silymarin (m/z = 483) and sodiated silymarin (m/z = 505) can be identified. HPBCD appears in several peaks according to the degree of substitution of the cyclodextrin ring with hydroxypropyl groups (m/z = 1215-1505), the average degree of substitution is 3. Silymarin-HPBCD complex can be detected also on the spectra in the 900-1100 range as double charged sodiated silymarin-HPBCD complex peaks [silymarin + HPBCD + 2Na]⁺. In the 600-800 region HPBCD peaks with double charge can be seen. These results clearly show that HPBCD is able to form stable complex with silymarin in water.

---

**Fig. 1. Silymarin-HPBCD mass spectra**
Effect of cyclodextrins on silibinin solubility in silymarin-cyclodextrin complexes

The low aqueous solubility of silymarin is responsible for its moderated absorption from the intestine. Several trials were performed to improve its solubility and bioavailability (Yanyu et al., 2006, He et al., 2007, Chen et al., 2005). Beta-cyclodextrins were also used to improve these properties (Arcari et al, 1992), but comparative studies on the effects of cyclodextrin derivatives are not available. In the present study we compared the solubilisation effect of HPBCD, RAMEB, DIMEB and QABCDP on silymarin. For this we dissolved silymarin-cyclodextrin complexes in water to obtain 0.5 mg/ml silymarin concentration in the solutions and determined the concentration of silibinin, the main component of silymarin, in the solution by HPLC. Fig. 2. shows that all of the cyclodextrins are able to solubilise silibinin in water and there are no differences among their solubilisation ability.

Another problem with silymarin solubilisation is that it is difficult to achieve high-concentration solutions in water. We prepared different solutions with increasing the amount of silymarin-HPBCD complexes to get 1, 2 and 10 mg/ml silymarin concentrations in the solutions and determined the silibinin concentrations by HPLC. Fig. 3 shows that increasing the silymarin-HPBCD concentration, the silibinin concentration increases proportionately in the solution. At 10 mg/ml we observed fine precipitation which had no influence on the silibinin solubilisation, but at 20 mg/ml the precipitation completely hindered silibinin dissolution (data not shown). Above 10 mg/ml silymarin concentration the 1:20 silymarin-HPBCD complex is not stable in water.
CONCLUSIONS
In order to increase the aqueous solubility of silymarin we prepared silymarin-cyclodextrin complexes using HPβCD, RAMEB, DIMEB and QABCDP. We used kneading method which is a simple and fast process of the formation of cyclodextrin complexes. Based on our results we can conclude that this method was efficient to produce silymarin-cyclodextrin complexes with each cyclodextrin. By using mass spectrometry we confirmed the existence of silymarin-HPβCD complexes in water. The solubility study of the complexes clearly shows that each cyclodextrin was able to solubilise silymarin in water and the concentration of silybin, the main component of silymarin, was almost the same in each sample. Increasing the dissolved amount of silymarin-HPβCD complexes the silybin concentration increased in the solutions up to 10 mg/ml silymarin concentration. Even if at the highest concentration we detected some precipitation, the silybin concentration was not decreased. The solutions were stable at lower concentrations, and they maintained also their stability upon dilution by water. This is due to the excess of cyclodextrins, because the complexes contained 20 times more cyclodextrin, which is 1:7.4 silymarin-cyclodextrin molar ratio. We can conclude that we formulated stable silymarin-cyclodextrin complexes with improved solubility which are suitable for further biological and pharmacological examinations.

ACKNOWLEDGEMENTS
This work was financially supported by the Hungary-Romania Cross-Border Co-operation Programme 2007-2013 (HURO/0901/058/2.2.2.) and TÁMOP-4.2.2/B-10/1-2010-0024.

REFERENCES
Shamama Javed, PhD; Kanchan Kohli; Mushir Ali Reassessing Bioavailability of Silymarin Altern Med Rev 16(3) (2011) pp. 239-249


Solubility increasing experiments of silymarin with cyclodextrins