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

The Development Of Internal And Topical Pharmaceutical Dosage Forms Containing Silybum Marianum, In Vitro And In Vivo Investigations

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The Examination takes place at the Library of Department of Pharmacology, Faculty of Pharmacy, University of Debrecen, 13th of October 2017, at 11:00

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The PhD Defense takes place at the Lecture Hall of Bldg. A, Department of Internal Medicine, Faculty of Medicine, University of Debrecen, 13th of October 2017, at 13:00

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1. Introduction

Silybum marianum (SM) has been successfully applied for the treatment of various liver disorders including acute and chronic viral hepatitis, alcoholic liver diseases, toxin and drug induced hepatitis and cirrhosis, fatty liver radiation, and toxicity. The topical use of silymarin has also received attention because of its antioxidant, anti-inflammatory and immunomodulatory properties, which may prevent UVB-induced skin damages or chemically induced skin disorders including erythema, photoaging and skin cancer.

The therapeutic effect of the plant is due to a flavonolignan-complex called silymarin,

However, the therapeutic effects of silymarin are restricted due to its poor water solubility resulting in low oral absorption and bioavailability after oral administration. These factors also influence the topical use of silymarin.

During the extraction process of Silybum marianum seeds two phases, a solid powder and an oil extract can be reached. A number of approaches have been used to increase its solubility and thereby bioavailability of the powder extract of silymarin. These include complexation with cyclodextrin and phospholipids, incorporation in solid dispersion, solid lipid nanoparticles and also formulation of self-(micro) emulsifying drug delivery system S(M)EDDS. Most of these studies deal with the internal and external effect of the powder, but not with the therapeutic effect of the native silymarin oil. SEDDS and SMEDDS are composed of poorly soluble drugs, lipoids, surfactants and co-surfactants and they are capable of forming oil-in-water emulsions upon gentle agitation provided by the gastrointestinal motion. The spontaneous formation of a microemulsion advantageously presents the lipophilic drug in a dissolved form, and the resultant small droplet provides a large interfacial surface area for drug release and absorption.

However, the low solubility and low bioavailability of silymarin limit extensive topical applicability. Many methods and dosage forms have been described for optimizing these parameters. Surfactants as penetration enhancers are able to influence the barrier integrity of the skin and potentiate the transport mechanisms of active pharmaceutical ingredients. However, just some articles deal with the potential of well-designed carrier systems, but the liberation and absorption of active pharmaceutical ingredients may be affected by using of ideal topical carriers. Due to the relative impermeability of the stratum corneum,

extensive preformulation studies are necessary in order to develop topical vehicle with optimal drug release and skin penetration.

During our experiments we worked with the solid powder and the oil phases extracted from the seeds of Silybum marianum.

From the silymarin oil we developed oral SMEDDS formulation in order to investigate it's hepatoprotective effect. From the silymarin powder we formulated an o/w emulsion type cream in which silymarin powder was in dissolved form and we used penetration enhancers as emulgents in the formulations. The creams containing silymarin powder in dissolved form enhanced skin penetration of the active substance and the compositions also showed antioxidant effect against UV radiation.

2. Aim of work

Our aim was to formulate an internal and external dosage form from the powder and the oil extract of Silybum marianum seed.

I. In the first part of the experiments internal dosage forms were developed from silymarin oil. Our aim was to formulate a stabile SMEDDS, in which the oil phase is the active substance as well in the preparations and also to improve the hepatoprotective effect of this dosage form.

According to these points we proposed the following experimental plan:

1. To formulate and investigate stabile SMEDDS containing silymarin oil with proper droplet size by using the suitable tensides, co-tensides.
2. To examine and prove the hepatoprotective effect of SMEDDS containing silymarin native oil. The in vivo investigations of the formulation in mice, to determine plasma ALT and AST and antioxidant enzymes from liver homogenate and to examine liver histology.

II. In the second part of our experiments creams were formulated from silymarin powder. Our aim was to dissolve silymarin powder in the topical formulations and to choose the proper penetration enhancers in order to increase the penetration of the active substance. The therapeutic target was to prepare a cream with antioxidant effect in order to cure and prevent skin disorders caused by UVB radiation.

1. Optimizing the dissolution of silymarin powder.
2. Formulate an o/w emulsion type cream containing silymarin powder with penetration enhancers.
3. Investigate the consistency of creams with Texture Analysis and perform in vitro dissolution studies with Franz diffusion cell.
4. Perform in vitro cytotoxicity test on HeLa and HaCaT cell line.
5. Design in vivo irritancy test and determine TEWL values on guinea pig model.
6. Investigate the antioxidant activity of creams on HaCat cells in vitro and on guinea pig model in vivo against UVB radiation.
7. Investigate the HO-1 enzyme levels from guinea big skin tissues in the pre- and posttreatment of silymarin creams against UVB radiation.

3. Materials and Methods

3.1. Materials

Transcutol HP, Capryol 90 és Labrasol was a gift from Gattefossé (Lyon, France) Cremophor RH40, Cremophor A6, A25 was obtained from BASF (Ludwigshafen, Germany). Sugar esters were offered by Sisterna Company (Roosendaalc, Holland).
HeLa cells (human cervical cancer cells) were obtained from the European Collection of Cell Cultures (ECACC, Public Health England, Salisbury, UK). HaCaT cells (human keratinocyte cells) were obtained from Cell Lines Service (CLS, Heidelberg, Germany).
All other reagents were obtained from Sigma-Aldrich (Budapest, Hungary)

3.2. The origin of Silybum marianum seeds

The seeds of milk thistle were from Arad (Romania). Plants were supplied by a farmer, voucher specimens already exist at the Faculty of Natural Sciences, Vasile Goldis Western University of Arad.

3.3. Extraction of Silybum marianum seed oil and powder

Silymarin oil and powder was extracted from the seeds of Silybum Marianum at the Department of Applied Chemistry, University of Debrecen according to the method of Kahol et al. During the extraction process of Silybum marianum two phases, a solid and an oil extract can be reached. Solid silymarin powder was obtained after drying the solid phase. From the liquid hexane extract, hexane residue was evaporated from the extract under vacuum in a rotary evaporator and the reached yellow silymarin oil was used in our experiments. 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. The silymarin oil was characterized using Shimadzu QP-2010 GC-MS instrument.

3.4. Formulation of silymarin oil- SMEDDS, the investigation of therapeutic effect

3.4.1. Formulation of SMEDDS-SM oil

According to our preliminary studies Cremophor RH40, Labrasol, Capryol 90 and Transcutol HP have been selected for SMEDDS formulation. Self-Emulsifying combinations have been formulated by water dilution method with various previously mentioned tensides and co-tensides. Tenside components were mixed at 37 ° C by a Schott Tritronic dispenser combined with a Radelkis OP-912 magnetic stirrer. The applied concentrations of SM native seeds oil have been incorporated in the system at room temperature. To evaluate any signs of phase separation, the mixtures were equilibrated for 24 h. Erweka DT 800 rotating paddle apparatus (Erweka Gmbh, Heusenstamm, Germany) was used to evaluate the efficiency of self- emulsification of different mixtures. One gram of each mixture was added to 200 ml of distilled water with gentle agitation provided by a rotating paddle at 70 rpm and at a temperature of 37 ° C. The process of self-emulsification was visually monitored for the rate of emulsification and for the appearance of the produced emulsions. The visual properties registered against the increment of the applied tenside component in ternary triangular diagrams. Plotting points of preferential combinations selected according to Cartesian coordinate calculation.

3.4.2. Determination of droplet size of self-micro-emulsifying systems

The diameter of dispersed phase was investigated by a Cumulant Dynamic Light Scattering (DLS) device. To obtain the diffusion coefficient the intensity correlation function has been analysed. The measurements have been performed by a Brookhaven Fotometer apparatus. The operation temperature was adjusted to 25° C, the laser detection angle to 90 degree, Lambda to 533 nm, index to 1,334 by Particle Sizing Program 3.1. Diameters of dispersed droplets according to the diffusion coefficient have been evaluated automatically by the computer program.

3.4.3. Experimental animals

The hepatoprotective effect after carbon tetrachloride intoxication was examined on Swiss male mice. The animals were maintained at a 12 h light/dark cycle, at constant temperature. The animals had free access to food and tap water *ad libitum*, the weight of mice were 25 ± 3 g. All experimental procedures were approved by the Ethical Committee of Vasile Goldis, Western University of Arad. Throughout the experiments, animals were processed according to the suggested international ethical guidelines for the care of laboratory animals.

3.4.4. The treatment protocol of mice with silymarin oil-SMEDDS

48 animals were divided into six groups. The control (1st group) and the CCl4 group (2nd group) received only isotonic saline solution. Other groups were pre-treated with two different doses of silymarin oil-SMEDDS (500 mg/kg, 1000 mg/kg) during 7 days. On the 8th day the CCl4 control group and the 3rd (SMEDDS p.o. 500 mg/kg) and 4th (SMEDDS p.o. 1000 mg/kg) groups were injected with CCl4 (1ml/kg) intraperitoneally.

The mice were anaesthetized on 9^{th} day (narcotic: ketamin/xylazin 80/6 mg/ttkg) and blood was collected from venae cavae and animals were terminated via cervical dislocation.

3.4.5. Assay of plasmatic hepatic markers

The collected blood before cervical dislocation was placed in heparinized tubes and centrifuged for 15 min at 2000 g in order to obtain plasma samples which were used immediately to determine ALT and AST activities. The plasmatic activities of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were evaluated by the spectrophotometric method using commercially available kits (Roche reagents, France) according to the manufacturer's indication.

3.4.6. Preparation of protein extract from liver tissue

For the preparation of the total protein extract, 0.1 g of liver tissue was suspended in a cold 0.1 M Tris-HCl buffer (pH 7.4) containing 5 mM EDTA and a freshly added pro- tease inhibitor cocktail, and homogenized for 2 min at 16 Hz using a ball mill (type MM 301, Retsch GmbH & Co, Haan, Germany). The homogenate was centrifuged at 8,000 rpm for 30 min at 4 ° C to remove the cell debris. The supernatant was collected for enzymatic analyses (SOD, CAT, GPX, GR), as well as GSH, MDA assay.

3.4.7. Histopathology

Liver specimens were fixed in 4% phosphate buffered formalin, embedded in paraffin. 5 μm thick sections were cut to the examinations that were stained with hemoxycillin using a standard procedure. Frozen sections were cut to 8 μm thick with the SLEE MNT cryotome for Oil Red O staining. Samples were fixed in 10% buffered formaldehyde and stained with Oil Red O kit (Bio-Optica) according to the manufacturer' instructions. Mounted slides were examined under a light microscope (Olympus BX43 microscope) and photographed using a digital camera (Olympus XC30).

3.4.8. Liver antioxidant status

3.4.8.1. Determination of Superoxide dismutase (SOD) from liver tissue

SOD activity was determined using a spectrophotometric method based on the decrease of optical density at 340 nm due to NADH oxidation by the generated superoxide anion. One unit of enzyme activity is the amount of enzyme required of NADH oxidation inhibition rate of 50%.

3.4.8.2. Determination of Catalase (CAT) from liver tissue

CAT was detected spectrophotometrically at 240 nm, by monitoring H_2O_2 decomposition. The CAT activity was expressed as U/mg protein. One unit of enzyme decomposes one μmole of H_2O_2 in a minute at 25°C and pH7.

3.4.8.3. Determination of Glutathione peroxidase (GPX) from liver tissue

GPX activity was measured according to Beutler's method, through spectrophotometrically changes at 340 nm due to oxidation of NADPH to $NADP^+$ by tert-butyl hydroperoxide. The concentration of NADPH was calculated using a molar extinction coefficient of $6.22\times103 M^{-1}\bullet cm^{-1}$ and the activity was expressed as U/mg. One U of activity is defined as that quantity of enzyme responsible for the oxidation of one μmole of NADPH per minute.

3.4.8.4. Determination of Glutathione reductase (GR) from liver tissue

GR activity was assayed by the decrease in the optic density at 340 nm, due to NADPH oxidation, as result of the enzymatic reduction of the oxidized glutathione (GSSG). The activity of this enzyme was expressed as U/mg. One unit of enzyme oxidizes one μmole of NADPH in a minute in defined conditions. The enzymatic activities were reported to protein concentration, and expressed as % of controls.

3.4.8.5. Determination of GSH concentration from liver tissue

GSH was detected in tissue homogenate after deproteinization with 5 % sulfosalicylic acid using the Detect X® Glutathione colorimetric detection kit, according to manufacturer's instructions. The method involved a kinetic analysis in which amounts of GSH caused a continuous reduction of DTNB reagent 5,5'-dithiobis(2-nitrobenzoic acid) which forms a yellow complex with the reactive, but not protein SH groups. The formed yellow complex was quantified at 412 nm using a 10 mM GSH calibration cure. The GSH levels were calculated as nmoles/mg protein.

3.4.8.6. Lipid peroxidation assay

Hepatic lipid peroxidation was assayed by a fluorimetric method described by Del Rio et al. and expressed in terms of malondialdehyde (MDA) content. The liver homogenate (200 μL) was incubated with 700 μL of 0.1 M HCl for 20 min at room temperature. A volume of 900 μL of 0.025 M thio- barbituric acid was added, and the mixture was incubated at 37

° C for 65 min. Further, the samples were subjected to fluorescence analysis (λ_{ex} = 520 nm; λ_{em} = 549 nm) (Spectrofluorimeter FP-6300 JASCO) and the concentration of malondialdehyde was estimated using 1,1,3,3-tetramethoxypropane as standard. The results were expressed as nmoles of MDA/mg protein.

3.5. The formulation and examination of creams containing silymarin powder

3.5.1. Formulation of topical creams

For formulations different emulgents were used. Polysorbate 60, Cremophor and Sucrose Esters (SP50, SP70 and PS750). The oil-in water creams were produced by melting (60°C) cetostearyl alcohol, stearic acid and isopropyl miristate and mixed in order to prepare the oil phase of the preparation. The aqueous phase containing propylene-glycol, emulsifying agent, and purified water was heated up to the same temperature (60°C) and mixed with the oil phase, homogenized and cooled down to 25°C. Then, Nipagin M was mixed in the preparation and finally, the active substance, SM. Final concentration of the powder incorporated in the creams was 5% either in suspended form (compositions I-V) or in dissolved form, previously dissolved with the help of Transcutol (compositions VI-X).

3.5.2. Dosage form examination of creams

3.5.2.1. Consistency examinations of creams

The resistance of cream formulations was measured by CT3 Texture analyser (Brookfield, Middleboro, U.S.A). Compression test as normal test was performed. The following parameters were fixed: trigger load (4g), target (10 mm), speed (0,50 mm/s). TA5 Cylinder type probe (12,7 mm diameter, and 35 mm length) was used during the test. The resistance of creams was expressed as N.

3.5.2.2. In vitro examinations with Franz diffusion cell

Membrane diffusion and permeability studies were performed with a vertical Franz-diffusion cell system (Hanson Microette TM Topical and Transdermal Diffusion Cell System). 0.3 g of sample were placed as donor phase on cellulose-acetate membrane (pore size 0.45 μm). Pre-treatment of the membrane by soaking in IPM was performed. Effective diffusion surface area was 1.767 cm2. 30% (v/v) alcohol was used as an acceptor phase in order to enhance the solubility of silymarin. Rotation of the magnetic stirrer was set to 450 rpm. The receptor medium was thermostated at 32±0.5 °C throughout the experiment to imitate the temperature of physiological skin on the membrane in the Franz cell. Experiments were performed for 6 hours. Samples of 0.8 mL were taken from the acceptor phase and replaced with fresh receiving medium. Quantitative measurement of silymarin was carried out with an UV- spectrophotometer (Shimadzu Spectrophotometer, Japan, Tokyo) at a wavelength of λ=287 nm. Calibration curve was determined before the spectroscopic measurements of silymarin. Linear connection was found between the concentration of silymarin and the measured absorbance. All experiments were performed in quintuplicates.

3.5.3. Cell cultures

HeLa cells were grown in plastic cell culture flasks in DMEM (Dulbecco's Modified Eagle's Medium), supplemented with 3.7 g/L NaHCO3, 10% (v/v) heat-inactivated fetal bovine serum (FBS), 1% (v/v) non-essential amino acids solution, 1% (v/v) L-glutamine, 100 IU/mL penicillin, and 100 IU/mL streptomycin at 37 °C in an atmosphere of 5% CO2. HaCaT kerinocytes were grown in DMEM supplement with FBS (7%, v/v), streptomycin (100 U/ml), penicillin (0,1 mg/ml) and glutamine (4 mmol/l) in a humidified atmosphere with CO2 (5%; v/v) at 37 ºC.

Culture medium was changed twice a week for both cell types under laminar air flow box. Cells were seeded at a density of 1×10^5 cells/cm ^{2} and grown near to confluence for experiments. The passaging of cells were done under laminar air flow box after they were grown to confluence. For cytotoxic and antioxidant experiments, cells were used between passage numbers 20 and 40.

3.5.4. MTT- assay

Cytotoxic effect of compositions I.-X. was evaluated by a colorimetric cytotoxicity method i.e. the MTT test. HaCaT keratinocytes and HeLa cell lines were used for evaluation of the in vitro cytotoxic effects. The test was performed as follows: HaCaT and HeLa cells were seeded on flat bottom 96-well tissue culture plates at a density of 10^3 cells/well and allowed to grow in a CO_2 incubator at 37 °C for 7 days, until they were grown to confluence. Culture medium was removed at the 3rd day under laminar air flow box. For these studies, the culture medium was removed, test solutions were added, and the cells were incubated for a further 30 min. at 37°C. After removing the samples, the cells were washed twice with 1 ml PBS, and another 3-hour-incubation in a medium containing MTT at the concentration of 0.5 mg/ml followed. The purple formazan crystals were dissolved in acidic isopropanol (isopropanol: 1.0 N hydrochloric acid = 25:1). Absorbance was measured at 570 nm against a 690 nm reference with FLUOstar OPTIMA Microplate Reader (BMG LABTECH, Offenburg, Germany). Cell viability was expressed as the percentage of the untreated control. Each experiment was repeated five times with five wells for each concentration.

3.5.5. Experimental animals used for in vivo experiments

Male Hartley guinea-pigs weighing 250-350 g were used for evaluation of dermal antioxidant effects and for the determination of HO-1 enzyme. The animals were fed regular rodent chow ad libitum with free access of water. The guinea-pigs were allowed 1 week to acclimatize before the experiments and were kept at 25±2°C, with a relative humidity of 55±5% and a 12 h light-dark cycle. All animals included in the present study were handled and received humane care in compliance with the National Society for Medical Research and Guide for the Care and Use of Laboratory Animals prepared by the national Institutes of Health (No. 10/2014/DEMÁB, extended until 2019)

3.5.6. In vivo skin irritation test

Before in vivo antioxidant tests, formulations (III, IV, VIII, IX) were tested for skin irritation. TEWL value was measured before and after 24 h application of the selected formulations to the dorsal skin of guinea-pig. 0.1 g cream was spread uniformly over a sheet of non-woven polyethylene cloth (2 cm x 2 cm), which was then applied to the back area of a guinea-pig. Before the experiment the dorsal skin of animals were shaved properly. The polyethylene cloth was fixed with Tegaderm 3M ® adhesive dressing (3 Med Kft, Budakeszi, Hungary) and Fixomull® stretch adhesive tape (MEDIGOR Bt, Veszprém, Hungary). After 24 h, the cloth was removed, and the treated skin area was cleaned with a cotton wool swab. After withdrawal of the vehicle for 30 min, TEWL of the applied skin was measured with Tewameter® (TM300, Courage& Khazaka, Köln, Germany) and results were compared to those values that were measured before 24 hours of the treatment. The temperature and relative humidity in the laboratory were kept at 25º C and 55%, respectively. The sample number for each experiment was five (n=5).

3.5.7. UV-B irradiation on HaCaT cells, pre-treatment and post-treatment of cells.

HaCaT cells were seeded on flat bottom 12-well tissue culture plates at a density of 1 x10 ^{5} cells/well and allowed to grow in a CO_2 incubator at 37 °C for 7 days, until they were grown to confluence. Culture medium was removed at the 3rd day under laminar air flow box. UV-B irradiation was an acute exposure, one irradiation of 350 μW from a distance of 20 cm.

In the pre-treatment group, culture medium was removed, test solutions were added, and the cells were incubated for further 20 min. (37 °C). After UV-B irradiation of 10 min. samples were removed, cells were washed with PBS and incubated for 24 hours (37 °C). In the post-treatment group, cells were first irradiated for 10 min., then treated with samples for 20 min. washed with PBS and incubated for 24 hours (37 °C). The following parameters were used for monitoring the antioxidant capacity of different compositions on UVB treated keratinocytes: SOD, CAT, GPx and malondialdehyde (MDA) activities. All experiments were performed in quintuplicates.

3.5.8. UV-B irradiation, pre-treatment and post-treatment with silymarin cream formulations, in vivo

Pre-treatment and post-treatment with SM in different compositions of creams (III, IV, VIII, IX) were tested for antioxidant capacity before and after UV-B radiation. Animals were exposed to UV-B irradiation (acute exposure, one irradiation of 350 μW) from a distance of 20 cm. At the beginning of the experiment dorsal skin of guinea-pigs was shaved and covered with aluminium foil into which ten rectangular slots (0.8 x 1.3 cm) had been cut, next 0.1 g cream was applied to the skin under each slot. For the antioxidant and HO-1 enzyme studies animals were divided into two groups of 10 animals each. Guinea-pigs in group I were pre-treated topically with the cream formulations for 20 min., then the animals were placed under UV-B apparatus and the uncovered skin was irradiated for 10 min. Creams were washed 24 hours after irradiation and skin samples were collected. In the II. group, animals were first irradiated for 10 min., then post-treated topically with the cream formulations and after 24 hours creams were washed and skin samples were collected. At the end of experiment, animals were anesthetized with ketamine/ xylazine (30-44 mg/kg, 1-5 mg/kg) and terminated via cervical dislocation. Tissue samples were dissected. Samples were perfused and rinsed with a PBS (phosphate buffered saline pH 7.4) solution to remove any red blood cells and clots. Skin samples were frozen with liquid nitrogen and kept at -80ºC until further processing.

3.5.9. Superoxide dismutase activity

Tissues were homogenized (Homogeniser, X520, Ingenieurbüro CAT, M-Zipperer GmbH, Germany) in 20 mM HEPES buffer (1mM EGTA, 210 mM mannitol, and 70 mM sucrose /g tissue), pH 7.2. Then homogenate was centrifuged at 1500 X g for 5 minutes at 4º C, and the supernatant assayed for SOD activity using the Cayman assay kit (Cayman Chemical, Ann Arbor, MI, USA; www.caymanchem.com/pdfs/706002.pdf)

Cells (1 x 10^6) were collected using a rubber policeman, centrifuged at 1000 X g for 10 minutes at 4º C. Cell pellet was homogenized in cold 20 mM HEPES buffer (1mM EGTA, 210 mM mannitol, and 70 mM sucrose /g tissue), pH 7.2 centrifuged at 10,000 X g for 15

minutes at 4ºC, and the supernatant assayed for SOD activity using the Cayman assay kit (Cayman Chemical, Ann Arbor, MI, USA; www.caymanchem.com/pdfs/707002.pdf)

3.5.10. Catalase activity

Tissue samples were homogenized (Homogeniser, X520, Ingenieurbüro CAT, M-Zipperer GmbH, Germany) in cold buffer (50 mM potassium phosphate, pH 7.0, containing 1 mM EDTA) per gram tissue, centrifuged at 10000 X g for 15 minutes at 4ºC, and the supernatant assayed for CAT activity using the Cayman assay kit (Cayman Chemical, Ann Arbor, MI, USA; www.caymanchem.com/pdfs/707002.pdf)

Cells (1 x 106) were collected using a rubber policeman, centrifuged at 1,000 x g for 10 minutes at 4º C. Cell pellet was homogenized on ice in 2 ml cold potassium phosphate buffer, pH 7.0, containing 1 mM EDTA per gram tissue, centrifuged at 10000 X g for 15 minutes at 4ºC, and the supernatant assayed for CAT activity using the Cayman assay kit (Cayman Chemical, Ann Arbor, MI, USA; www.caymanchem.com/pdfs/707002.pdf)

3.5.11. Glutathione peroxidase activity

Tissue samples were homogenized (Homogeniser, X520, Ingenieurbüro CAT, M-Zipperer GmbH, Germany) in 10 ml cold buffer containing 50 mM Tris-HCL, pH 7,5, 5 mM EDTA and 1mM dithiothreitol, per gram tissue. The homogenate was then centrifuged at 10000 X g for 15 minutes at 4ºC. The supernatant was assayed for GPx activity using the Cayman assay kit (Cayman Chemical, Ann Arbor, MI, USA; www.caymanchem.com/pdfs/703102.pdf)

Cells (1 x 106) were collected using a rubber policeman, centrifuged at 1000 x g for 10 minutes at 4º C. Cell pellet was homogenized in cold buffer containing 50mM Tris-HCL, pH 7.5, 5 mM EDTA and 1mM dithiothreitol. The homogenate was then centrifuged at 10,000 X g for 15 minutes at 4ºC. The supernatant was assayed for GPx activity using the

Cayman assay kit (Cayman Chemical, Ann Arbor, MI, USA; www.caymanchem.com/pdfs/703102.pdf)

3.5.12. Lipid peroxidation (MDA) Test

Tissue samples (10 mg) and cells (1 x 106) were homogenized on ice in 300 µl of the MDA Lysis Buffer containing 3 µl of BHT (100 x) centrifuged at 13000 X g for 10 minutes to remove insoluble material. The supernatant was assayed for Lipid Peroxidation (MDA) assay kit (Sigma-Aldrich, St.Louise, USA). Lipid peroxidation was determined by the reaction of MDA with thiobarbituric acid (TBA) to form a colorimetric (532 nm) product, proportional to the MDA present. All antioxidant tests were performed in quintuplicates.

3.5.13. Measurement of HO-1 enzyme activity

Guinea pig skin tissue samples were homogenised in 200 mM phosphate buffer. pH 7.4. The supernatant was collected by centrifugation of the homogenate for 30 minutes at 20.000 g at 4º C. Assessment of heme oxygenase activity was measured on each sample of supernatant according to protocol used by Tenhunen et al (1968). Briefly, enzyme activity was performed with a computer-based spectrophotometric analysis of heme formation to bilirubin. HO-1 enzymatic assay used a reaction mixture containing, aliquot of the supernatant, plus glucose-6-phosphate 2mM, glucose-6-phosphate dehydrogenase 0,14 U/ml, heme 15 μM, NADPH 105 μM, rat liver cytosol as a source of recombinant biliverdin reductase 120 μg/ml, MgCl2 2mM and KH 2PO4 100mM. The reaction was made up to a final volume (2ml) for each sample and was incubated at 37ºC for 1h in the dark. The reaction was arrested by placing the samples on ice. Chlorophorm was added to terminate the reaction and bilirubin was extracted following centrifugation, and measured by spectrophotometric method, reading the difference in absorbance between 460 and 530 nm. The HO-1 activities were expressed in nmol of bilirubin formed per 1 milligram of protein per hour.

3.6. Statistical analysis

Results of SMEDDS experiments were analysed using GraphPad Prism 5 software. Statistical significance between the experimental groups were assessed by two-way ANOVA and Bonferroni post-test. Values were expressed as mean ± standard deviation (SD). The values of significance were evaluated with “P values”. $P \leq 0.05$ was considered statistically significant.

Results of experiments with creams containing silymarin powder were analysed using SigmaStat (version 3.1; SPSS, Inc.) and presented as means ± SD. Comparison of the groups in MTT cell viability assays one-way ANOVA and Tukey’s test had been performed.

In case of SOD, CAT, MDA, GPx and HO-1 enzyme activity evaluations Repeated-Measures Anova and for quantifying associations between groups Pearson correlation had been performed. Significant differences were indicated with asterisks and/or crosses in our designed figures. Differences were regarded as significant, with $p < 0.05$. Experiments on HeLa and HaCaT cells were repeated ten times (n=10), all other experiments were carried out in quintuplicates and repeated at least five times (n=5).

3.7. Contributions

The in vivo animal experiments of SMEDDS containing silymarin were performed with the help of Vasile Goldis Western University of Arad. (Ardelean Aurel, Costache Marieta, Dinischiotu Anca, Hermenean Anca)

The in vivo animal experiments of creams containing silymarin were performed with the help of Pharmacology Department, University of Debrecen (Dr. Juhász Béla, Dr. Bombicz Mariann, Dr. Priksz Dániel, Dr. Varga Balázs)

The HO-1 enzyme measurement was performed with the help of Dr. Csaba Varga form University of Szeged.

The evaluation of statistical analysis was performed with the help of Dr. Zoltán Ujhelyi

The rest of experimental methods and the evaluations have been done by the author.

4. New scientific results of thesis

4.1. The results of the formulation and investigations of SMEDDS-silymarin oil preparations.

In our novel SMEDDS formulation studies silymarin oil was the active substance and the lipid/oil part of the system. Several reported that the property of oil component and the weight percentage of oil in SMEDDS formulation are determining factors in these preparations. Formulations may enhance the oils own therapeutic effect and the oral absorption as well.

Besides the selection of oil, surfactant and co-surfactant as well as the mixing ratio of oil to surfactant/co-surfactant may play an important role in SMEDDS formulation. In our preparation Cremophor RH 40, Transcutol HP were the surfactants. Labrasol, Capryol 90 as non-ionic tensides were the co-surfactants. Biological effects of several nonionic amphiphilic surfactants have been also investigated according to their chemical structure. In our previous studies, the possibility of enhanced bioavailability by cell monolayer structural alteration of various surface-active agents has been shown. Various surface-active agents could improve both the paracellular and transcellular uptake of active ingredients in vitro as well. Presumably, our preparation may also increase the oral bioavailability of silymarin oil by the alteration of intestinal cell monolayer.

According to Batakov (2001), the oil obtained from the seeds of Silybum Marianum produced an antioxidant effect on liver tissues of rats poisoned with CCl_4. The oil just in high doses (2000mg/kg) reduced the level of lipid peroxidation, increased catalase activity but did not reduce the concentration of selenium in the liver (which decreased as a result of CCl_4 intoxication). The oil did not increase the activity of superoxide dismutase in liver tissues. In spite of this study, we found that silymarin oil has its own hepatoprotective therapeutic effect. The application of SMEDDS increased the effectiveness and decreased the dose of silymarin oil significantly (500 mg/kg, 1000 mg/kg).

In our study, liver protection was achieved by oral application of two doses of silymarin oil SMEDDS (500 mg/kg, 1000 mg/kg) before CCl_4 toxic administration.

The decrease in serum aminotransferases activity by the high- dose of SMEDDS in CCl_4-

intoxicated mice indicates that this silymarin oil formulation preserves the structural integrity of hepatocellular membrane, which was supported by the histological findings.

In the present study, the pre-treatment with SMEDDS-silymarin oil followed by a single dose administration of CCl_4, generated antioxidant protection for mice hepatocytes, evidenced by increased antioxidant enzyme activities (SOD, CAT and GPX) compared to the intoxicated group, in which they were lower compared to control.

We suppose that this situation appeared because, possibly, the oxidation of endogenous GSH in the presence of linoleic acid (an important constituent of Silybum marianum seed oil used for SMEDDS obtaining) catalysed by lipoxygenase was accompanied by superoxide anions generation. Moderate levels of superoxide anions could increase the SOD reaction rate and produce more hydrogen peroxide, which could be decomposed in the reactions catalysed by GPX and CAT. Also the rise of GR activity could be possibly due to conjugated linoleic acid isomers as previously Choi et al. have demonstrated.

Preventive treatment with SMEDDS obtained from Silybum marianum seed oil restored the specific activities of all enzymes in the individuals exposed to CCl_4 in a dose dependent manner. The significant elevation of MDA, the end product of lipid peroxidation, was generally considered a marker of formation of free radicals. The decrease of SOD, CAT, GPX and GR activities in the individuals exposed to CCl_4 have generated a lower antioxidant defence capacity in their liver and as a result MDA concentration increased significantly. The pre-treatment by SMEDDS decreased this parameter in the liver of intoxicated mice, which might suggest their antioxidant capacity, which could be due to the presence of tocopherol and ascorbic acid 2,6 dihexadecanoate, present in silymarin seeds oil.

4.2. The results of formulation and investigation of creams containing silymarin powder

In our investigation o/w emulsion type creams containing silymarin powder were formulated with proper consistency.

Beside those preparations that contained the silymarin powder in suspended form, we could formulate creams with dissolved silymarin powder. Silymarin powder was dissolved in

Transcutol which is a good solubilizing agent and a very strong penetration enhancer, but it is non-toxic and biocompatible with the skin.

Different non-ionic, amphiphilic tensides were selected for our ten compositions: P 60, CRC, Sucrose stearate SP50, SP70, and SP750 to develop optimal carrier systems and promote SM penetration via the skin. These surfactants may play a role in the solubilisation and may also modify the bioavailability of drugs. In vitro release study provided information about SM dissolution from the carrier systems. Release rate differences were observed among the formulations, compositions with Sucrose esters SP 50 and SP 70 resulted in the highest diffused silymarin values. Outcomes of texture analysis studies confirmed that these creams showed optimal consistency values. Although, these surfactants resulted in good diffusion values determination of safety and non-toxic concentration is important for their application.

Biocompatibility investigations (MTT-test) were performed on HeLa and HaCaT cells to verify the tolerability of cream compositions. HeLa cells represent a reliable model for testing topical preparations while HaCaT cells are suitable to assess skin irritancy potential of excipients. In the MTT cell viability test, sucrose-esters SP50 and SP 70 proved to be more tolerable than Polysorbates and Cremophors in compositions. Compositions containing these tensides (SP 50 and 70) did not reduce HeLa and HaCaT cell viabilities under 50 %. Since in vitro cytotoxicity data alone not necessarily predicts in vivo issues irritancy tests were also performed. Results of animal skin irritancy test verified that our topical preparations containing silymarin, different surfactants and TC were highly tolerated, but evaluated of the data of preformulation studies (in vitro dissolution study, texture analysis study, in vitro MTT-cell viability tests), four compositions (III, IV, VIII and IX) were selected for further in vivo antioxidant tests on HaCaT cells and in guinea pigs.

Topical application of silymarin causes depletion of catalase and induction of cyclooxygenase in mouse model: application of these preparations to mouse skin prevents UV-induced oxidative stress. In the present study, it was observed that every preparations containing Sucrose stearate SP50 or SP 70 increased CAT, SOD and GPx and decreased MDA activities compared to the controls. Post-treatment of suspended and dissolved silymarin produced the same effect as pre-treatment with different compositions. Dissolved

silymarin in Transcutol (VIII., IX.) caused a more significant increase in the activity of ROS eliminating enzymes in the case of post-treatment. Reduction in the levels of end products of lipid peroxidation were registered after treatment with these four (III., IV., VIII., IX.) compositions. Treatment with compositions VIII and IX. resulted in the greatest increasing of ROS species, however the level of GPx was the highest in the case of pre and post-treatment. It can be explained that penetration of dissolved silymarin powder in Transcutol was better than compositions III. and IV. containing suspended silymarin powder. Higher solubility of silymarin powder may influence the penetration and the efficacy of topical formulations. GPx is a non-enzymatic antioxidant and maintain pro-oxidant/anti-oxidant balance resulting in cell and tissue stabilization. Those formulations that contain sucrose esters SP 50 and SP70 may promote all ROS species in a different rate but the promotion of non-enzymatic GPx may be more intensive. It was assumed that our in vitro data presented tight correlation with in vivo parameters as there were an increase in activity of CAT, SOD and GPx levels compared to the controls. Elevations in the levels of end products of lipid peroxidation were also measured on HaCaT cells. Svobodova A. et al. 2007 confirmed that the flavonolignan components of silymarin dissolved in DMSO suppressed oxidative stress caused by UVA and was useful in the treatment of UVA-induced skin damage. Silymarin showed a dose dependent protective effect against UV-induced damage in human keratinocytes via inhibition of NF-κB activation. Higher levels of SOD and CAT were measured on HaCaT in the case of pre and post-treatment than in guinea pig. These enzymatic ROS level activations may be more expressed on keratinocytes due to the different effect of surfactants. The same tendency was observed on Caco-2 cell lines treated by blends of surfactants due to the modulation of tight junction proteins. Different correlations were estimated between in vitro cell culture experiment data and in vivo animal experiment.

Marked decrease in the ROS species may refer UVA and UVB-induced oxidative stress. However, the work of Katiyar.et al. showed that topical application of silymarin (dissolved in alcohol) inhibits UVB-induced inflammatory responses and photocarcinogenesis in mice as well. Nevertheless, high increase of HO-1 enzyme levels also represents stress against different environmental factors (i.e. UVB-induced photodamages, cellular stressors. Both UVA and UVB irradiation can lead to high levels of HO-1 expression of

cells and it is involved in an adaptive protective response against oxidative damage and it is considered a marker of cellular oxidative stress. In our study, high increase of HO-1 enzyme activities were measured after UVB irradiation. Interestingly, topical administration of silymarin creams did not affect the HO-1 activity compared to the untreated control. It might be supposed that the high activity of antioxidant enzymes by the rapid elimination of free radicals may influence the activation of HO-1 enzyme systems in the skin. Silymarin powder supplemented with penetration enhancers may be a potential in topical anti-inflammatory and antioxidant therapy to the skin based on the impact of ROS species activation and moderation of HO-1 enzyme activity.

5. Summary

In the **first part** of our experiments SMEDDS preparation containing silymarin oil was formulated in which the oil component was also the active substance.

We determined that the silymarin oil-SMEDDS dosage form diminished hepatic enzyme levels and elevated antioxidant enzyme levels in mouse liver tissue against carbon tetrachloride intoxication.

The hepatoprotective effect of SMEDDS containing silymarin oil was also verified by histopathological investigations on mouse liver tissue after carbon-tetrachloride toxication. Our results showed that silymarin oil-SMEDDS compositions prevent carbon-tetrachloride induced hepatotoxicity presumably by the inhibition of lipid peroxidation and the membrane stabilization of hepatocytes.

To summarize our investigations we can conclude that per oral use of silymarin oil SMEDDS preparations have hepatoprotective effect.

In the **second part** of our work o/v creams containing silymarin powder and penetration enhancers were formulated with appropriate consistency. The results of in vitro membrane diffusion studies justified that Transcutol – which also helped in the dissolution of silymarin powder- with sugar-ester type emulgents elevated the amount of active substance across the diffusion membrane.

The pre and post-treatment of creams containing silymarin powder in dissolved form showed sufficient antioxidant activity against UV-induced oxidative stress in guinea pig model and HaCaT cells. Significance of post-treatment is outstanding because silymarin creams may provide alternative potentials against solar UV radiations. In vitro and in vivo data did not showed total correlation during our experiments. It can be concluded that evaluation of in vitro data alone is not sufficient but complemented with in vivo animal experiments may anticipate that possibility of human clinical trials.

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7. Publications

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Registry number: DEENK/163/2017.PL
Subject: PhD Publikációs Lista

Candidate: Pálma Fehér
Neptun ID: A8GZI9
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List of publications related to the dissertation

1. **Fehér, P.**, Ujhelyi, Z., Váradi, J., Fenyvesi, F., Róka, E., Juhász, B., Varga, B., Bombicz, M., Priksz, D., Bácskay, I., Vecsernyés, M.: Efficacy of Pre- and Post-Treatment by Topical Formulations Containing Dissolved and Suspended Silybum marianum against UVB-Induced Oxidative Stress in Guinea Pig and on HaCaT Keratinocytes.
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List of other publications

3. Váradi, J., Harazin, A., Fenyvesi, F., Réti-Nagy, K., Gogolák, P., Vámosi, G., Bácskay, I., **Fehér, P.**, Ujhelyi, Z., Vasvári, G., Róka, E., Haines, D. D., Deli, M. A., Vecsernyés, M.: Alpha-Melanocyte Stimulating Hormone Protects Against Cytokine-Induced Barrier Damage in Caco-2 Intestinal Epithelial Monolayers.
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