

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

Formulation and investigation of Self-Emulsifying Drug Delivery System
(SEDDS) containing natural herb extract or different antitumor agents

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1. Introduction

Approximately 70-75% of medications marketed worldwide are administered *per os* and are proven to be less effective than desired. The majority of newly developed drugs represent poor aqueous solubility and stability. Drug instability results in lack of dose appropriateness after oral administration. Many different methods have been found to enhance drug dissolution rate and improve the physicochemical stability of drugs by applying surface-active agents, Cyclodextrins, nanoparticles or liposomes. Lipid-based drug delivery systems represent one of the most popular technologies for improving oral bioavailability and solubility. Micro- and nanoemulsions are lipid-based formulations that have a significant potential for drug delivery applications and self-micro/nanoemulsifying drug delivery systems (SM/NEDDS) considered as the best of these systems. Many studies suggested that SM/NEDDS are suitable carrier systems for drugs which are very sensitive to hydrolysis. SM/NEDDS are characterized as isotropic mixtures of synthetic or natural oil and solid or liquid surface active agents or, alternatively, a single or several hydrophilic solvents and co-solvents and a drug which spontaneously forms oil-in-water (o/w) nanoemulsion/microemulsion droplets with water following a gentle stirring. In gastrointestinal fluid, this system spreads readily in the GI lumen and the agitation necessary for self-emulsification is provided by the gastric and intestinal digestive motility. After dilution with aqueous media, the oil droplets keep the drug inside of them or they form a micellar solution due to the very high surface-active agent concentration of such formulations. The drug will be delivered to the GI mucosa in dissolved state by these fine droplets; therefore, it will be readily available for absorption and its efficacy and bioavailability will also be increased. Micro/nanoemulsions improve oral bioavailability, protect drugs from enzymatic hydrolysis and have high drug solubilization capacity and outstanding thermodynamic stability.

The phenomenon of self-emulsification occurs only when specific combinations of pharmaceutical excipients are present.

Several chemotherapeutic agents are used in the treatment of cervical cancer. Cisplatin is considered to be among the most effective drugs that treat advanced uterine and cervical cancer. Bleomycin sulfate is a mixture of glycopeptide antitumor antibiotics that has a unique mechanism of antitumor activity in cervical cancer. Ifosfamide is an anti-cancer drug and is widely used in the treatment of cervical, ovarian and testicular

malignancies. In recent chemotherapy, cisplatin, bleomycin and ifosfamide (BIP) in combination has also been applied against inoperable cervical cancer. To decrease the serious side effects resulted from use of cytostatic medications it is necessary to formulate modern drug delivery systems which can reduce toxicity by decreasing the dose of potent therapeutic agent. SMEDDS are frequently used to increase the bioavailability of poorly soluble drugs by presenting and maintaining API in a dissolved state, in small droplets of oil with a ability to penetrate through various biological barriers.

Plantago lanceolata has been widely used for medical purposes, such as the treatment of bleeding and tissue damage, antioxidant, anti-inflammatory, antibacterial antiviral and hepatoprotective. Some prominent pharmacological studies are outlined in the following section. Iridoid glucoside aucubin and its derivatives have been identified as important biologically active compounds in plantain by several studies. The main bioactive component of *Plantago lanceolata* is verbascoside (acteoside), which is a phenylpropanoid glycoside. However the high capability of its bioactive components for hydrolysis resulted in poor stability of this natural extract. SNEDDS is frequently used for the stabilization of natural products and these carrier systems may also increase the bioavailability of natural bioactive materials.

2. Aim of work

The main purpose of this research is to prepare SM/NEDDS formulations for oral bioavailability enhancement of a poorly stable water soluble drug due to hydrolysis which diminish drug absorption and also to investigate the inhibitory potential of different antitumor agents in SMEDDS carrier on human cervical cancer HeLa cells.

The specific objectives of the thesis were to:

- Identify oil and surfactant combinations and that can solubilise over 15-20-% water and pseudoternary phase diagrams with a large micro – (nano) emulsion areas
- Investigate the phase behaviour of selected SMEDDS/SNEDDS upon dilution with water and evaluate the droplet size controlled by Dynamic Light Scattering Method (DLS)
- Evaluate the biocompatibility and toxicological profile of SMEDDS compositions on different cell lines. MTT cytotoxicity test was done to certify the cytocompatibility of these samples.

In the first part of the thesis, we also want to certify the applicability of SMEDDS compositions containing different antitumor agents on HeLa cells. The inhibitory effect of antitumor agents on HeLa cells was checked in the presence of inflammatory mediators (IL-1- β , TNF- α) as an *in vitro* model of inflamed human cervix.

In the second part of the thesis, Plantago lanceolata extract was prepared and was formulated in different SNEDDS compositions. The specific objective of these formulations was to improve the stability of bioactive components of Plantago lanceolata extract in SNEDDS compositions. Antioxidant activity by DPPH assay and antiinflammatory activity of Plantago lanceolata SNEDDS compositions by ear edema test were evaluated to prove the higher activity of formulated compounds.

3. Materials and Methods

3.1. Materials

3.1.1. SNEDDS components

3.1.1.1. SNEDDS components for the formulations of antitumor agents

Labrasol, Capryol 90, Lauroglycol FCC and Transcutol HP were kind gifts from Gattefossé, Lyon, France. Kolliphor RH 40 were obtained from BASF, Ludwigshafen, Germany.

3.1.1.2. SNEDDS components for the formulations of *Plantago lanceolata* extract

Labrasol was used as selfemulsifying and solubilizing agents in our formulations in addition to increasing the intestinal absorption of drug and was purchased from Gattefossé SAS (Lyon, France). Kolliphor RH or Hydrogenated Castor Oil (Cremophor RH 40) was obtained from BASF Chem Trade GmbH (Limburgerhof, Germany). Highly purified diethylene glycol monoethyl ether (Transcutol HP) was purchased from Gattefossé (Lyon, France). Labrasol or Kolliphor RH 40 as surfactant, and Transcutol HP, Capryol 90, Lauroglycol FCC as co-tensides were used in our compositions. Isopropyl myristate used as oily phase/solvent and obtained from Merck company (Darmstadt, Germany).

3.1.2. Active pharmaceutical ingredients in the compositions of different SNEDDS

3.1.2.1 Antitumor agents

Anticancer drugs as Cisplatin (*cis*-Diammineplatinum(II) dichloride), Bleomycin sulfate,

Ifosfamide (*N*,3-Bis(2-chloroethyl) tetrahydro-2*H*-1,3,2-oxazaphosphorin-2-amine-2-oxide) were obtained from FLUKA Analytical Ltd. (Seelze, Germany).

3.1.2.2. Dry *Plantago Lanceolata* leaf methanolic extract

The leaves of the *P. lanceolata* base on pharmacopoeial quality were commercial source. The dried leaves of *P. lanceolata* chopped and milled into a uniform powder prior to further work.

The fine powder was extracted with MeOH under reflux followed by filtration process at the Department of Pharmacognosy, University of Debrecen. Afterwards the herb extract was defatted with hexane and dried again. After dissolving dry extract with MeOH 10mg/mL solutions were prepared and diluted for dissociation of sample. Accurate standards of bioactive components of Catalpol, Aucubin and Acteoside were used as standards to develop the calibration curves. The quantification of natural products by LC-MS was run on a Thermo Accela HPLC attached to a Thermo LTQ XL Linear Ion Trap MS.

3.1.3. Cell culture models

3.1.3.1. HeLa cells

HeLa (human cervical cancer cells) was obtained from the European Collection of Cell Cultures (ECACC, Public Health England, Salisbury, UK). Cells were grown in plastic cell culture flasks in Dulbecco's Modified Eagle's Medium (Sigma-Aldrich Buchs, St Gallen, Switzerland), 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. The cells were routinely maintained by regular passaging. For cytotoxic and transport experiments, cells were used between passage numbers 20 and 40. The culture media was replaced with fresh media in every 72 h.

3.1.3.2. Caco-2 cells

Caco-2 (human adenocarcinoma cancer cells) was obtained from the European Collection of Cell Cultures (ECACC, Public Health England, Salisbury, UK). Cells were grown in plastic cell culture flasks in Dulbecco's Modified Eagle's Medium (Sigma-Aldrich Buchs, St. Gallen, Switzerland), 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. The cells were routinely maintained by regular passaging. For cytotoxic and transport experiments, cells were used between passage numbers 20 and 40. The culture media was replaced with fresh media in every 72 h.

3.1.4. Inhibitory Effect of Different SNEDDS formulations Containing Antitumor Agents in the Presence of Inflammatory Mediators

The inhibitory effect of different SMEDDS containing cytostatic drugs was evaluated in the presence of inflammatory mediators on human cervical cancer HeLa cells. HeLa cells were plated in 96-well sterile plates, at a density of 10⁴ cells per well in 100 mL of medium, and incubated with 1.25 µL IL-1-β (0.1 µg/µL) and 3.75 µL TNF-α (0.1 µg/µL) for 3–4 h. After ignition, HeLa cell proliferation test was used.

3.1.4.1. Inflammatory mediators

IL-1-β, TNF-α have been purchased from Sigma-Aldrich.

3.2. Methods

3.2.1. SNEDDS preparations

3.2.1.1. Formulation of Self-Nano-Emulsifying Drug Delivery Systems containing BIP

Water and oil dilution method had been applied for preparation of 6 different SMEDDS formulations in combination with different surfactants and co-surfactants that their compatibility and solubility were screened formerly. Surfactants and co-surfactant were mixed at 37 °C by Schott Tritronic dispenser (SI Analytical, Mainz, Germany) combined with Radelkis OP-912 magnetic stirrer (Radelkis, Budapest, Hungary). The applied concentrations of chemotherapeutic agents were dissolved in the systems at room temperature by constant agitation. To evaluate any signs of phase separation, the mixtures were equilibrated for 24 hour. An Erweka DT800 rotating paddle apparatus (Erweka GmbH, Heusenstamm, Germany) was used to evaluate the efficiency of self-emulsification of different mixtures. One gram of each composition was added to 200 mL of distilled water under gentle agitation condition 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 surfactant component in Ternary triangular diagrams. Plotting points of preferential combinations were selected according to cartesian coordinate calculation.

3.2.1.2. Formulation of Self-Nano-Emulsifying Drug Delivery Systems containing *Plantago Lanceolata* extract

Different self-emulsifying combinations have been formulated by the water and oil dilution method with various previously tested surfactant and co-surfactant. The compositions are presented in Table 4. Surfactant and co-surfactant were mixed at 37 °C by Schott Tritronic dispenser (SI Analytical, Mainz, Germany) combined with Radelkis OP-912 magnetic stirrer (Radelkis, Budapest, Hungary). The required amount of *Plantago lanceolata* herb extract was dissolved in the systems at room temperature by permanent agitation. To evaluate any signs of phase separation, the mixtures were equilibrated for 24 hour. An Erweka DT800 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 condition 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 surfactant component in Ternary triangular diagrams. Plotting points of preferential combinations were selected according to cartesian coordinate calculation.

3.2.2. Investigation of SNEDDS compositions

3.2.2.1. Droplet size and Zeta potential of SNEDDS containing APIs

Diameter of dispersed phase was investigated by a Dynamic Light Scattering device (Malvern, Worchetershire, UK). The cumulant Dynamic Light Scattering (DLS) method was used for determination of droplet size of formulated emulsions. To obtain the diffusion coefficient the intensity correlation function has been analyzed. The measurements have been performed by Brookhaven Photometer (Brookhaven,

Upton, NY, USA). During the operation temperature was 25 °C, the laser detection angle was adjusted to 90°, Lambda (λ) 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. To evaluate the zeta potential the samples were diluted with 10 mL distilled water by gentle agitation at room temperature. Zeta potential of samples had been evaluated by Zetasizer NanoZS analyser. Measurements were performed in quadruplets to obtain an average and standard deviation of the results.

3.2.2.2. In Vitro Dissolution Test of PL-SNEDDS

In vitro dissolution test of PL-Compositions based on the determination of DPPH Radical Scavenging Activity of *P. lanceolata* extract. In vitro drug release from PL-SNEDDS were conducted according to FDA-recommended dissolution methods in pH = 6.8. The dissolution condition was 500 mL of pH 6.8, Phosphate buffer at a paddle speed of 75 rpm. Aliquots of 3 mL were withdrawn and filtered using 0.45 μ m membrane filter predetermined time intervals of 5, 10, 15, 30, 60 min. The volume removed from each solution was replaced immediately with fresh dissolution medium. The determination of diffused *P. lanceolata* extract based on the DPPH Radical Scavenging Activity.

3.2.3. Biocompatibility evaluations on different cell lines

3.2.3.1. MTT cell viability assay on HeLa cell line with SNEDDS compositions containing antitumor agents

To exclude any toxic effect of the blank SNEDDS and their components on HeLa cells, MTT cell viability test was used. Cells were seeded on flat bottom 96-well tissue culture plates at a density of 10^4 cells/well and allowed to grow in a CO₂ incubator at 37 °C for 4 days. For these studies, the culture medium was removed, surfactant or SNEDDS solutions were added, and the cells were incubated for a further 30 min. After removing the samples, another 3-h-incubation in a medium containing MTT at the concentration of 0.5 mg/mL followed. The dark blue formazan crystals were dissolved in acidic isopropanol (isopropanol: 1.0 N hydrochloric acid = 25:1). The 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.

3.2.3.2. MTT cell viability assay on Caco-2 cell line with SNEDDS compositions containing *Plantago lanceolata* extract

To exclude any toxic effect of the blank SNEDDS and PL-SNEDDS on Caco-2 cells, MTT cell viability test was used. Cells were seeded on flat bottom 96-well tissue culture plates at a density of 10⁴ cells/well and allowed to grow in a CO₂ incubator at 37°C for 4 days. For these studies, the culture medium was removed, surfactant or SNEDDS solutions were added, and the cells were incubated for a further 30 min. After removing the samples, another 3-h-incubation in a medium containing MTT at the concentration of 0.5 mg/mL followed. The dark blue formazan crystals were dissolved in acidic isopropanol (isopropanol:1.0 M hydrochloric acid = 25:1). The 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.

3.2.4. Investigation of the activity of SNEDDS compositions

3.2.4.1. Inhibitory Effect of Different SNEDDS Containing Antitumor Agents

HeLa proliferation were also evaluated by using MTT cell viability assay. Cells were plated in 96-well sterile plates, at a density of 10⁴ cells per well in 100 mL of medium, and incubated for 3–4 h. Cytostatic drugs alone in cell culture medium or incorporating in SMEDDS were prepared immediately before use and added in a volume of 50 µL and total volume of 200 µL (with 150 mL fresh medium supplement) per well at final concentrations of cytostatic drugs between 5×10^{-4} and 5 mg/mL. After 72 h the samples were removed and 100 mL of freshly diluted MTT solution at a concentration of 0.5 mg/mL, was pipetted into each well and the plate was incubated for 3 h at 37 °C in a humidified, 5% CO₂ atmosphere. After a specific period, cell viability was evaluated by measurement of the absorbance at 520 nm, using a FLUOstar OPTIMA Microplate Reader (BMG LABTECH). All experiments were made in quadruplicate. Standard deviations were $\leq 10\%$.

3.2.4.2. Inhibitory Effect of Different SNEDDS Containing Antitumor Agents in the Presence of Inflammatory Mediators

The inhibitory effect of different SNEDDS containing cytostatic drugs was evaluated in the presence of inflammatory mediators on human cervical cancer HeLa cells. HeLa cells were plated in 96-well sterile plates, at a density of 10^4 cells per well in 100 mL of medium, and incubated with 1.25 μL IL-1- β (0.1 $\mu\text{g}/\mu\text{L}$) and 3.75 μL TNF- α (0.1 $\mu\text{g}/\mu\text{L}$) for 3–4 h. After ignition, the previously described HeLa cell proliferation test was used.

3.2.4.3. DPPH Radical Scavenging Activity of SNEDDS-PL Samples

Each sample (PL-Composition 1–3, 5–8, Composition 1–3, 5–8, PL-E) was reacted with the stable DPPH radical in ethanol (96%). The reaction mixture consisted of adding 100 μL of sample, 900 μL of absolute ethanol, and 2 mL of DPPH radical solution (0.06 mM) in absolute ethanol. The mixtures incubated for 30 min. When DPPH reacted with an antioxidant compound, which can donate hydrogen, it was reduced. The reaction resulted in color change from deep violet to light yellow. Quantitative measurement of remaining DPPH was carried out with an UV-spectrophotometer (Shimadzu Spectrophotometer, Tokyo, Japan) at a wavelength of $\lambda = 517$ nm. In case of photometric determination mixtures, absolute ethanol served as background. The control solutions were the same compositions without *P. lanceolata* extract. To demonstrate the improved antioxidant effect of combinations, blank *P. lanceolata* extract (10 mg/mL) was applied as well. The scavenging activity percentage (AA% = Antioxidant Activity) was determined according to Mensor et al.

3.2.5. In vivo animal models

3.2.5.1. Animals and Experimental Groups

Swiss male mice (22 ± 3 g), supplied by the Animal House of the School of Medicine, Faculty of Pharmacy, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran, (Ethical approval number is IR.AJUMS.REC. 1394.139) were used. The animals were maintained at a 12 h light/dark cycle, at constant temperature, with access to food and tap water ad libitum. All experimental procedures were approved by the Ethical

Committee of Ahvaz Jondishapur University of Medical Sciences, Faculty of Pharmacy, Ahvaz, Iran. During the experiments, animals were processed according to the suggested international ethical guidelines for the care of laboratory animals. 54 animals were used for hepatic function markers investigations and forty-eight animals for ear oedema tests. For the first experiment, the mice were divided into nine groups (one control and eight groups for SMEDDS-PL investigation), for the second experiment, eight mice groups were composed (one positive and one negative control and six groups for SMEDDS-PL investigation). The extracts (150 mg/kg/day) were administered by gastric gavage (100 μ L three times daily). The controls were given the same volume as in the test group.

The mice were anaesthetized on the final day of experiments, and blood was collected from venae cavae before mice were euthanized by cervical dislocation.

3.2.5.2 Preparation of Blood Plasma

The collected blood was placed in heparinized tubes and centrifuged for 15 min at 2000 rpm in order to obtain plasma samples, which were used immediately to determine ALT and AST activities.

3.2.5.3. Dimethyl-Benzene-Induced Inflammation Model

Anesthesia was induced by thiopental in an amount of 50 mg/kg intraperitoneally (i.p.), repeated as required. The posterior area of the right ear was then injected with 3m/m % dimethyl-benzene solution. This treatment was applied 30 min after the oral gavage. Thus, the oral administration of SNEDDS-PL was performed firstly, and the induction of inflammation was carried out secondly.

3.2.5.4. In vivo measurement of epidermal thickness changes on mice:

Measurement of Ear Oedema

The thickness of ear was measured by a micrometer caliper (Oxford Precision, Leicester, UK), with 0.1 mm accuracy before dimethyl-benzene treatment and 15 min after the first dimethyl-benzene application, then by each hour during a 6 h period after each 3m/m % dimethyl-benzene treatment according to Ujhelyi J., et al. SNEDDS-PL treatment was performed 30 min before starting time of ear edema induction. Data were

expressed in micrometers.

3.3. Statistical analysis of SNEDDS compositions

3.3.1. Statistical analysis in BIP-SNEDDS

Data were analyzed using SigmaStat (version 3.1; SPSS, IBM Inc, New York, NY, USA) and presented as means \pm SD. Comparison of groups was performed by one-way ANOVA. This ANOVA was used to compare the differences of each values belong to certain concentrations in MTT. We marked the significant differences with asterisks in figures. After that, the results among the groups were presented by Tukey's test. Differences were regarded as significant in case of $p < 0.05$. All experiments were carried out in triplicates and repeated at least three times.

3.3.2. Statistical analysis in PL-SNEDDS

Data were handled and analyzed using Microsoft Excel 2013 and SigmaStat 4.0 (version 3.1; SPSS, Chicago, IL, USA, 2015), and herein presented as means \pm SD. Comparison of results of MTT cell viability assays, hepatic function markers (AST, ALT), free radical scavenging activity test, in vitro dissolution test, and ear edema test was performed with one-way ANOVA and repeated-measures ANOVA followed by Tukey or Dunnett post testing. Difference of means was regarded as significant in case of $p < 0.05$. All experiments were carried out in quintuplicates and repeated at least five times ($n = 5$).

3.4. Contributions

Characterization of the Plantain and extract preparation have been done with the help of Dr. Gábor Vasas, Department of Pharmacognosy, University of Debrecen.

The evaluation of statistical analysis was performed with the help of Dr. Rudolf Gesztelyi Department of Pharmacology, University of Debrecen.

The in vivo animal experiments of SNEDDS containing *Plantago lanceolata* were performed with the help of Dr. Anayatollah Salimi from Ahvaz Jundishapur University of Medical Sciences.

DLS experiments have been conducted by Dr. Akos Kuki, Department of Applied Chemistry, University of Debrecen.

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

4. New Scientific results of thesis

4.1. The Result of the formulation and investigations of SNEDDS containing different Antitumor agents in the absence and presence of inflammatory mediators

Cervical cancer is the second most common cause of cancer-related deaths in women across the globe. Although, due to gynecological screening, the incidence rate of cervical cancer has decreased, it is still a priority to find the most effective and appropriate antiproliferative treatment. SNEDDS have attracted great attention as they enhance *per os* bioavailability, allow reducing the dosage, improve drug absorption profiles, help selectively target drug(s) towards specific absorption windows in the gastrointestinal tract and protect drug(s) from the intestinal environment. Lipid based drug delivery systems can be formulated successfully only if the lipid excipients are selected properly. In this investigation, the growth inhibition of human cervical cancer HeLa cells by six different SNEDDS formulations was studied; these SMEDDS formulations contained anticancer drugs—cisplatin, bleomycin sulfate and ifosfamide—alone and in combination. SNEDDS can potentially carry more than one API as delivery systems. In cervical cancer treatment, both monotherapy and/or combination of chemotherapeutic agents has been used efficiently but application of topical dosage formulations of antitumor agents seems to be more beneficial. Here, we have successfully developed SNEDDS of low dose combinations of cytostatic agents in order to increase the bioavailability and efficiency. Self-nanoemulsifying drug delivery systems as lipid-based carriers can enhance the solubility of poorly soluble APIs and are also able to modify the permeability of various drugs due to the large amounts of incorporated surface active agents which help enhance penetration. We screened the toxicity and permeability of SNEDDS and cytotoxicity of the compositions. Based on the results, these ingredients/compositions are not toxic within the concentration range we applied. The cells might have remained metabolically intact and the inhibition of their proliferation was not a result of cytotoxic effects of SNEDDS. In vitro paracellular and transcellular uptake of active ingredients could be improved by surfactants and co-surfactants which further increase the oral

bioavailability of certain APIs. Many studies confirmed the benefits of SNEDDS in peroral applications but the topical efficacy of SNEDDS formulations is still to be investigated. The efficacy of antitumor agents loaded in SNEDDS formulations was higher than alone. The most effective inhibition was achieved by applying composition 3 (1:2:12:6:2 ratio of Isopropyl myristate, Kolliphor RH40, Capryol 90, Transcutol HP and Labrasol). The inhibitory effects of these agents were proven to be additive, and when applied in SNEDDS, significantly more efficient than alone. In a randomized-controlled study, BIP was approved for advanced and recurrent cervical carcinomas. The *in vivo* study revealed that using nanoformulation for the treatment of cervical cancer cells is advantageous considering safety and potency aspects. There is a linkage between inflammation and cancer; since inflammation is a critical component of cervical cancer, it can stimulate tumor progression. In this experiment, we also investigated the effects of different SNEDDS compositions on human cervical cancer HeLa cells in the presence of inflammatory mediators IL-1- β and TNF- α . The results confirm that the efficiency of inhibition decreased under this condition. This evidence indicates that the advantage and efficiency of these carrier systems is hindered when inflammation is present.

4.2. The results of formulation and investigations of SNEDDS containing *Plantago lanceolata*

Plantago lanceolata has been widely used in herbal medicine due to its anti-inflammatory and antioxidant activities but its use is limited by the hydrolysis tendency of its bioactive components (acteoside, catalpol and aucubin) which makes the herbal extract unstable. According to Vertuani et al, 10% of the acteoside content can be lost at 40°C and pH=7 and 20% at pH=6. In case of parenteral administration, improvement of the stability of verbascoside can be achieved by liposomes which prevent its hydrolysis. Using self-nanoemulsifying drug delivery systems is a recent and effective method for enhancing the *per os* bioavailability of various poorly soluble drugs provided that the drug is potent and has high lipid solubility. This study revealed that PL-loaded SNEDDS could effectively enhance intestinal absorption of the moderately stable bioactive components in *Plantago lanceolata* by rapid self-emulsification and subsequent dispersion at the absorption sites. Our PL-SNEDDS compositions enhanced the free radical scavenging activity of *Plantago lanceolata* extract compared to a

positive control (non-formulated *Plantago lanceolata* extract). The reasons for selecting DPPH assay were its simplicity and reproducibility and the fact that the required chemicals were available in the laboratory. In addition, this assay is widely used to evaluate the antioxidant activity of plant metabolites. The therapeutic potential of *Plantago lanceolata* extract can be predicted but not sufficiently certified by DPPH reaction; therefore, ear inflammation assay was performed as well. Ear edema was induced by dimethyl-benzene and the outcome of the experiments proved that each PL-SNEDDS composition decreased the dimethyl-benzene-induced inflammation. The n-hexane-insoluble fraction of *P. lanceolata* exerted anti-inflammatory effects in mice. The fraction caused reduction in the volume of paw edema and COX-2 expression as well. The optimized formulation for antioxidant/anti-inflammatory and bioavailability assessments consisted of isopropyl myristate, Cremophor RH40/Labrasol and Transcutol HP. The *in vitro* dissolution rates of the active components of PL-SNEDDS compositions were significantly higher than those of *Plantago lanceolata* alone. On the basis of the predictive results of the DPPH test, an indirect dissolution experiment was designed. Higher level of free radical reduction was obtained for PL-SNEDDS compositions that contained 25–25% isopropyl-myristate–Kolliphor RH 40/Labrasol and 50% Transcutol HP. Both the dissolution and the ear inflammation test ranked the PL-SNEDDS compositions in the same order. In a study by Li et al, linear *in vitro*-*in vivo* correlation was found for a stable SNEDDS-persimmon leaf extract formulation. Lipid-based nanosystems can increase the efficiency of formulations by enhancing the bioavailability of drugs. The characteristics of the applied oil, surfactant and co-surfactant are essential factors in the development self-nano-emulsifying systems. In our SNEDDS formulation, we used Transcutol HP as co-surfactant which also enhanced penetration effectively, isopropyl-myristate as the oily phase which dissolved the lipophilic components of *Plantago lanceolata* and Kolliphor RH 40/Labrasol which enhanced the paracellular permeability of APIs in the Caco-2 cell monolayer and stabilized the most hydrophilic components of the plant compounds. Amphiphilic molecules are potentially ideal surface active agents and co-surfactants in microemulsions but to exclude toxicity, it is necessary to perform cytocompatibility screening. Therefore, we performed *in vitro* MTT cell viability tests for the evaluation of the toxicity of our SNEDDS compositions; in addition, *in vivo* AST/ALT levels were analyzed in mice. In our acute toxicity test, higher concentrations of PL-SNEDDS compositions were applied than in the cytotoxicity experiment on Caco-2 cells.

Nevertheless, the more diluted the composition, the safer it was. The samples with higher dilution (200 to 1000-folds) were found to exert no cytotoxic effects. These results have importance when assessing the toxicity profile of our compositions but they do not cover every aspect of this issue. Caco-2 cells are widely used for *in vitro* modeling of intestinal absorption and cytotoxicity because they allow rapid screening. Irritancy, potential and delayed toxicity of surface active agents can be predicted by *in vitro* assays but the results are more predictive when supported by *in vivo* tests (e.g. liver function tests in animals). Both tests ranked PL-SNEDDS compositions in the same order of toxicity with the exception of PL-SNEDDS compositions 4 and 8. These compositions were fatal to mice which indicate that overall toxicity is not predictable solely by MTT cytotoxicity testing. For the satisfactory determination of the toxicity profile of the compositions, it is necessary to perform more than one assay on different cell lines which has to be followed by *in vivo* animal toxicity studies. Still, the estimation of the risk factors and possible outcomes of human exposure requires carefully evaluated *in vitro* and *in vivo* data as well.

5. Summary

Micro- and nanoemulsions are simple, convenient and commercially viable new vehicles for delivery of active agents. They help enhance drug absorption and reduce systemic adverse effects. It is crucial in the formulation of micro/nanoemulsions to select excipients and evaluate safety appropriately, especially in the case of surface active agents and co-surfactants. It is known that micro/nanoemulsions can protect unstable drugs, increase drug solubility, improve absorption and bioavailability. Reducing droplet size results in faster and enhanced drug release and increased bioavailability in the case of certain APIs.

1. In both parts of our studies, SNEDDS formulations with different APIs content were prepared. The first six compositions contained antitumor agents, called BIP (bleomycin, Ifosfamide and cisplatin) combinations. The second eight SNEDDS contained natural herb extract, *Plantago lanceolata*. According to our previous works, biocompatible surfactant and co-surfactants were selected for the formulation of SNEDDS compositions. The cytotoxicity of formulated SNEDDS compositions were also controlled.
2. The average droplet size was evaluated by Dynamic Light Scattering method. Every average droplet size of compositions are in the nanometer range. The reason of higher droplet sizes was explained by the higher content of co-surfactant and the high HLB values of surfactants.

In the first part of the thesis:

- a. the topical applicability of SNEDDS compositions containing different antitumor agents on HeLa cells was proven. The inhibitory effect of mixed bleomycin sulphate, cisplatin and ifosfamide incorporated in SNEDDS was additive in IC₅₀ concentrations and significantly effective.
- b. Decreased inhibitory efficacy of cytotoxic drug-loaded SNEDDS compositions was confirmed in the presence of IL-1- β and TNF- α on human cervical cancer HeLa cells.
- c. Our experiments represent new finding because SNEDDS compositions on HeLa cells which is a reliable model for topical administration can enhance the effect of antitumor agents alone and in combination.

In the second part of the thesis:

- a.** New synthetic oils and surface active agents (surfactant and co-surfactant: i.e. Labrasol and/or Kolliphor RH 40 with high HLB values) can dissolve and stabilize unstable hydrophilic plant compounds by forming SNEDDS.
- b.** *Plantago lanceolata* containing SNEDDS compositions are potentially suitable for improving the stability of bioactive agents of *Plantago lanceolata* extract and are also capable to enhance the antioxidant and anti-inflammatory effects of plant compounds.

The practical relevance of the thesis is that lipid-based compositions can result in alternative ways of oral or topical applications of different APIs. Due to the penetration enhancer properties SNEDDS compositions can enhance the permeability of different active compounds. They can form small droplet size at the site of action and are able to show increased absorption compared to the other conventional oral or topical formulations.

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7. Publications



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Candidate: Azin Kalantari
Neptun ID:
Doctoral School: Doctoral School of Pharmacy

List of publications related to the dissertation

1. **Kalantari, A.**, Kósa, D., Nemes, D., Ujhelyi, Z., Fehér, P., Vecsernyés, M., Várad, J., Fenyvesi, F., Kuki, Á., Gonda, S., Vasas, G., Gesztelyi, R., Salimi, A., Bácskay, I.: Self-nanoemulsifying drug delivery systems containing *Plantago lanceolata*: an assessment of their antioxidant and antiinflammatory effects.
Molecules. 22 (10), 1-17, 2017.
DOI: <http://dx.doi.org/10.3390/molecules22101773>
IF: 2.861 (2016)
2. Ujhelyi, Z., **Kalantari, A.**, Vecsernyés, M., Róka, E., Fenyvesi, F., Póka, R., Kozma, B., Bácskay, I.: The Enhanced Inhibitory Effect of Different Antitumor Agents in Self-Microemulsifying Drug Delivery Systems on Human Cervical Cancer HeLa Cells.
Molecules. 20 (7), 13226-13239, 2015.
DOI: <http://dx.doi.org/10.3390/molecules200713226>
IF: 2.465





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List of other publications

3. Kalantar, H., Sabetkasaei, M., Shahriari, A., Haj Molla Hoseini, M., Mansouri, S., Kalantar, M., **Kalantari, A.**, Khazaei Poul, Y., Labibi, F., Moini-Zanjani, T.: The Effect of Rapamycin on Oxidative Stress in MCF-7 and MDA MB-231 Human Breast Cancer Cell Lines. *Jundishapur J. Nat. Pharm. Prod.* 11 (3), 1-6, 2016.
DOI: <http://dx.doi.org/10.17795/jjnpp-38177>

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The Candidate's publication data submitted to the IDEa Tudóstér have been validated by DEENK on the basis of Web of Science, Scopus and Journal Citation Report (Impact Factor) databases.

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8. Training courses and Conferences

Pharmaceutical Training Course, Research and Development, Compounding, Manufacture and Scale-up, Darou Pakhsh Pharma Chem Co. (DPPC), Tehran-Iran, September 2017.

Pharmaceutical Training Center, Group IMT, Tours-France, July-2017

APAX Business Development, Europe/USA, Rockway Consulting Company, Research work. August-December 2013.

7th BBBB International Conference on Pharmaceutical Sciences, Balatonfüred-Hungary, October-2017.

2nd European Conference on Pharmaceutics, Krakow-Poland, April 2017

Formulation, in Vitro and in Vivo investigations of Self-Micro Emulsifying Drug Delivery System containing natural herb extract

Clinical Pharmacology Conference, Debrecen-Hungary, December 2016

Formulation in Vitro and in Vivo investigation of Self-Emulsifying Drug Delivery System containing *Plantago lanceolata*

Clinical Pharmacology Conference, Debrecen-Hungary, November-2014.