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

Dr. Azin Kalantari

UNIVERSITY OF DEBRECEN

DOCTORAL SCHOOL OF PHARMACEUTICAL SCIENCES

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by **Dr. Azin Kalantari**

SUPERVISOR:

Dr. Ildiko Bacskay

UNIVERSITY OF DEBRECEN

DOCTORAL SCHOOL OF PHARMACEUTICAL SCIENCES

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1. AUTHOR's DECLARATION

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners. I understand that my thesis may be made electronically available to the public.

Azin Kalantari

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4. ABBREVIATIONS

API..... Active pharmaceutical ingredients

BCS……Biopharmaceutical Classification System

BIP .....Bleomycin,ifosfamide,cisplatin

CMC..... Critical Micelle Concentration

Cisplatin..... Cis-Diammineplatinum(II) dichloride

D..... Diffusion Coefficient

DE .....Dermal Edema

DMEM..... Dulbecco's Modified Eagle's Medium

DLS ..... Dynamic Light Scattering

DPPH ……2,2-diphenyl-1-picrylhydrazyl

EDTA ..... Disodium ethylenediaminetetraacetic acid

FBS .....Fetal Bovine Serum

GI ..... Gastrointestinal

HBSS.....Hanks' Balanced Salt Solution

HeLa..... Human cervical cancer cells taken from Henrietta Lacks

h..... Hour(s)

IPM.....IsopropylMyristateIfosfamide

IC50.....The half maximal inhibitory concentration

IL-1-β.....Interleukin-1beta

min.....Minute(s)

MTT.....3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

nm.....Nanometre or nanometer

OD.....optical density

o/w.....Oil in water

PBS.....Phosphate buffered saline

PEG 400.....Propylene Glycol 400

PL-SNEDDS.....Self Nanoemulsifying Drug Delivery systems containing Plantago Lanceolata extract

SD.....Standard Deviation

SEDDS.....Self Emulsifying Drug Delivery systems

SMEDDS.....Self Microemulsifying Drug Delivery systems

SNEDDS.....Self Nanoemulsifying Drug Delivery systems

TNF-α.....Tumour Necrosis Factor alpha

UV.....Ultraviolet

UV-VIS.....Ultraviolet Visible Spectroscopy

Z-Average.....Average Droplet Size

w/o.....Water in Oil

% w/v.....Percent Weight/Volume

5. INTRODUCTION

5.1. General characterisation of SMEDDS/SNEDDS

Approximately 70-75% of medications marketed worldwide are administrated *per os* and are proven to be less effective and useful than desired. The majority of newly developed drugs represent poor aqueous solubility and stability which makes it difficult to select the appropriate delivery system to be used in order to achieve satisfactory bioavailability of such medications, moreover, drug instability results in high intersubject and intrasubject variability accompanied by lack of dose appropriateness after oral administration (Kommuru TR,.et al, 2001). Many different methods and techniques have been found to enhance drug dissolution rate and improve the physicochemical stability of drugs by particle size reduction, complexation and applying surface active agents, cyclodextrins, nanoparticles or liposomes (Lian R., et al.2011). Recently, peroral formulations have gained increased attention; the efficacy of these drugs is dependent on their oral bioavailability which, in turn, depends on several factors for example the most important being drug solubility and stability in a hydrophilic environment and permeability across lipophilic biological membranes (K. Kohli, et al,.2010). In pharmaceutical formulations, emulsions are utilized as drug vehicles, particularly because they can potentially improve the peroral bioavailability of drugs with poor absorption profiles (Pouton CW et al., 1985). Using 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. Self micro/nanoemulsifying drug delivery systems (SM/NEDDS) have been considered as the best of these systems that can be produced by chemical rather than mechanical methods. (Barkat Ali Khan et al., 2012). SM/NEDDS are currently one of the commercially feasible formulation strategies for tackling drug stability and low bioavailability problems. SMEDDS and SNEDDS were investigated by several recent studies as promising candidates for enhancing the dissolution rate and bioavailability of drugs with poor solubility and stability (Deokate UA,.et al 2013). Many studies suggested that SM/NEDDS are suitable carrier systems for enhancing the absorption and solubility of drugs with poor water solubility and those drugs which are very sensitive to hydrolysis. Basically, 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 (Pouton C. Wet al.2000). When dispersed in the gastrointestinal (GI) fluid, this carrier 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 (Cho YD, Park YJ. 2013). Figure 1 represents a SM/NEDDS formulation pathway: (Paresh et al, 2014).

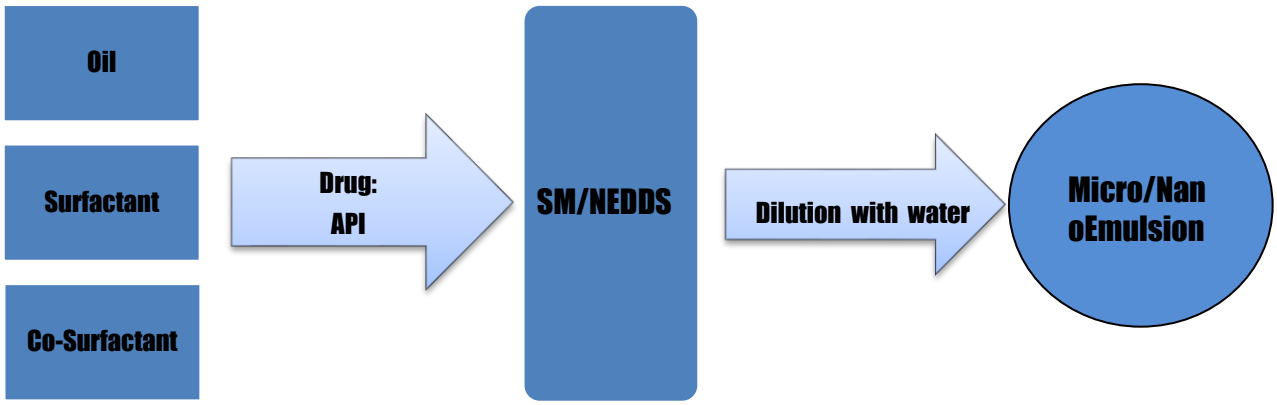


Figure 1: Schematic flowchart on the general formulation of self-micro/nano-emulsifying systems and their further conversion to transparent liquid of Micro/Nano emulsions.(Paresh et al, 2014)

SM/NEDDS should contain a low dose of the main component or the active pharmaceutical ingredient (API) which has to be sufficiently lipophilic; the aqueous phase interacts rapidly with the lipid matrix and forms fine particles of (o/w) micro/nanoemulsion. 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 (Jakki R,et al 2013).

After oral administration of SM/NEDDS in hard capsules, these preparations spread readily in the gastrointestinal tract. Gastric and intestinal motility provide additional agitation for self-emulsification and since the API is in dissolved state within small droplet diameter, absorption can take place through a large interfacial surface (Pouton CW,.et al. 2000).

The picture below shows a passageway of drug transport from SM/NEDDS carrier through lipophilic membranes (Zhang, L. et al 2016).

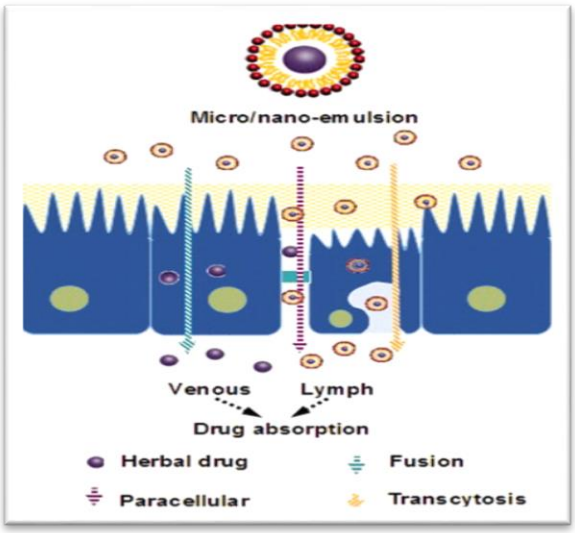


Figure 2: **Schematic diagram of mechanisms of intestinal drug transport from lipid-based formulations. (Zhang, L. et al 2016)**

Micro/nanoemulsions improve oral bioavailability, protect drugs from enymatic hydrolysis and have high drug solubilization capacity and outstanding thermodynamic stability. Unpleasant taste—which is caused by the lipid content and results in reduced patient compliance—is one of the difficulties with these carrier systems; furthermore, soft capsules cannot be used to encapsulate micro/nanoemulsions because of the water content of SM/NEDDS. (Patravale VB et al, 2003). SNEDDS/SMEDDS investigations present that lipid vehicles have great importance in the design and the success of drug delivery as they can control the rate of drug absorption and are digestible (Mittal Tanya et al, 2017).

5.1.1. Description of microemulsions

The word microemulsion was originally defined by Hoar and Schulman in the 1940s. They prepared a transparent, one-phase solution by titrating an opaque emulsion with hexanol (Fialho SL, et al, 2004). In SMEDDS, microemulsions are sometimes named as critical solutions due to their solubilization capacity and the frequently occurring optical reflection and fluctuation. They are bicontinuous systems, basically containing oil, water, a surface active agent, a co-surfactant and a drug. Microemulsions have ultralow interfacial tensions. They are typically intended for topical application and oral administration due to their high surface active agent concentrations. Microemulsions are isotropic, translucent, low-viscous, single phasic and thermodynamically stable liquid solutions (Talegaonkar et al., 2008).

SMEDDS form transparent microemulsions that are produced by the spontaneous emulsification method (phase titration method) and their formation is accompanied by different association structures (like oily dispersion, cubic, hexagonal, lamellar, and various gels, micelles and emulsion) on the basis of the chemical composition and the concentration of each constituent. The co-solvent content of SMEDDS is high but they contain less oil. In SMEDDS, the usual oil concentration is below 20%. Microemulsions can be separated into w/o or o/w types on the basis of whether they contain large amounts of oil or water (Talegaonkar, S.,et al 2008). Since the particle size of microemulsions is considerably smaller (< 300 nm) than the wavelength of visible light, microemulsions are transparent and no structures can be detected in these systems by optical microscope (Sumedha Nadkar,et al, 2010).

The most important difference between micro- and nanoemulsions is that microemulsions are nano-scale, spontaneously forming emulsions while nanoemulsions are nano-scale emulsions which can be produced by intense mechanical energy input. Aside from terminology, SNEDDS and SMEDDS are essentially different, considering thermodynamic stability. Table 1 presents the differences in physicochemical properties of SNEDDS and SMEDDS (Savale, S.K., et al 2015).

Physicochemical-Property	SNEDDS	SMEDDS
Appearance	Transparent in nature	Turbid in nature
Formation	Spontaneous formation	Vigorous shaking needed
Rate of absorption	Fast	Slow
Optimization	By Psedoternary phase diagrams	By ternary phase diagrams
Droplet size	>100 nm	200-300 nm
Stability	Thermodynamically and kinetically stable	Thermodynamically stable
Energy	Require low energy	Relatively high energy
Bioavailability	Maximum	Minimum

Table 1. Difference between SNEDDS and SMEDDS (Savale, S.K., et al 2015)

5.2.2. Description of nanoemulsion

In SNEDDS, nanoemulsion is defined as a colloidal particulate system in the submicron size range which acts as a carrier system and is manufactured to improve API delivery (Jaiswal M,et al. 2015). Nanoemulsions are nano-sized emulsions, generally consisting of oil droplets in water with diameters less than ∼200 nm (typically ranging from 20 to 100 nm). From a thermodynamic aspect, nanoemulsions arelike conventional emulsions (size > μm), in a non-equilibrium phase. On the other hand, in the case of nanoemulsions, the process of destabilization is prolonged (months); therefore, they can be considered kinetically stable. The main reason of this is their very small droplet size that prevents flocculation and coalescence. Two types of nanoemulsions—water-in-oil (W/O) or oil-in-water (O/W)—can be distinguished based on whether the water droplets are dispersed in oil, or vice versa.(Izquierdo P,et al.2002).

5.2. Composition and components of SM/NEDDS

The phenomenon of self-emulsification occurs only when specific combinations of pharmaceutical excipients are present. Several factors affect this process, such as the characteristics of the oil and the surface active agent, the ratio of these components, the concentration of the surface active agent and the temperature at which self-emulsification takes place. The first steps of SM/NEDDS formulation are identifying these specific excipient combinations and creating a phase diagram that presents different excipient concentrations at which self-emulsification occurs. In order to achieve a stable liquid formulation and an efficient self-emulsifying system, the mutual miscibility of the excipients to be used in SM/NEDDS formulation is also of great importance (Pouton CW,.et al, 2006). Considering chemical aspects, lipids are one of the most useful excipient classes available currently. Different lipid categories are accessible in the market which allow the formulator to select a suitable excipient flexibly, although the formulator should be careful when opting for a certain excipient. There are a few factors described by Pouton et al. which should be taken into consideration when choosing a lipid excipient. They are: (a) regulatory issues—irritancy, toxicity (b) solvent capacity (c) miscibility (d) morphology at room temperature (e) self-dispersibility (f) digestibility and the fate of the digested products (g) capsule compatibility (h) purity, chemical stability and (i) cost (Pouton CW 2006).

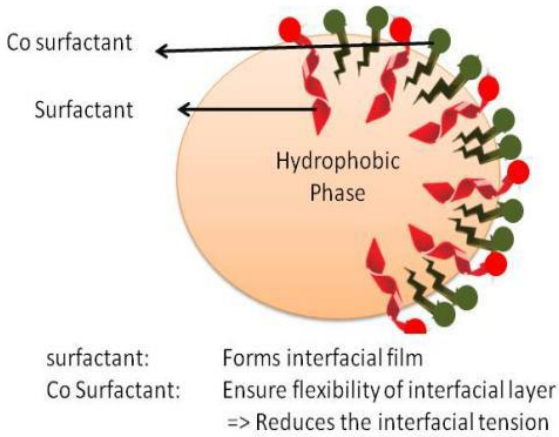
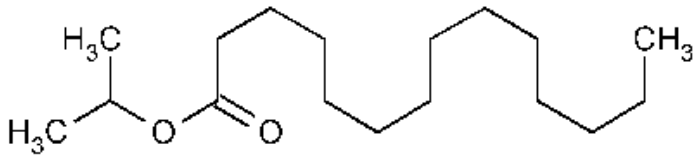


Figure 3: Micro/Nano emulsion Structure (Abhijit Dave,2012).

One of the most important excipients of SM/NEDDS formulations is oil. It is necessary not only for solubilizing the required amount of a lipophilic drug or facilitating self emulsification but it can also increase the fraction of the lipophilic drug that is transported via the intestinal lymphatic system which results in increased absorption from the gastrointestinal

tract. Hydrolyzed or modified vegetable oils have played an important role in the success of SM/NEDDS due to their formulation and physiological advantages. New semi synthetic medium-chain triglyceride oils have surface active properties and are frequently substituting the conventional medium-chain triglyceride (Khoo SM. et al., 1998).

Isopropyl myristate is a synthetic low viscosity oil with colorless and odorless properties. It is a commonly used excipient (lubricant or emollient) in pharmaceutical and cosmetic products. Isopropyl myristate is composed of isopropyl alcohol (a derivative of propane) and myristic acid (a common fatty acid). It is most frequently used as an ingredient in after shaves, hair care products, deodorants, antiperspirants, bath oils, toothpastes, mouthwashes, and a variety of lotions and creams. Another benefit of isopropyl myristate is that it can thicken formulations. Molecular formula: C17H34O2 (National Center for Biotechnology Information, 2017).



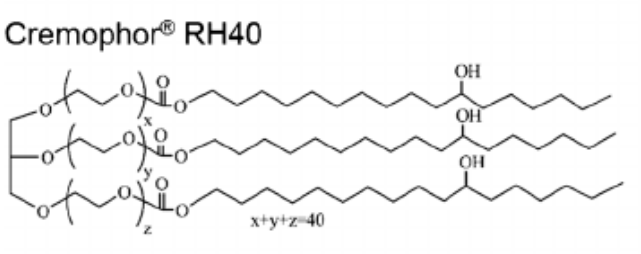
Formula 1. Chemical structure of isoprpyl.myristate

Surfactants are potentially irritant or poorly tolerated because they can penetrate biological membranes (so-called hydrophobic surfactant) or solubilise membrane components (hydrophilic surfactants). Generally, cationic surfactants are more toxic than their anionic counterparts which in turn are less tolerated than non-ionic surfactants. Non-ionic surface active agents which have high hydrophilic–lipophilic balance (HLB) values are less toxic than ionic surfactants as they are more compatible with biological systems and less affected by the pH and the ionic strength used in SM/NEDDS formulation (e.g. Cremophore, Labrasol, Labrafac, Tween, etc.) (D. J. Hauss, et al, 2007). In order to facilitate instantaneous generation of o/w droplets and/or rapid spreading of the SM/NEDDS formulations in an aqueous environment, high HLB surfactants are preferred. Surfactants are amphiphilic compounds which can solubilize or dissolve comparatively large amounts of hydrophobic drug compounds. This can prevent the drug from precipitation in the gastrointestinal tract and prolong the existence of drug molecules (Shah NH.et al, 1994). In order to have table SM/NEDDS, surfactant strength tipically ranges from 30 to 60% w/w of the formulation. The

presence of highly hydrophilic surfactants is essential for formulating SM/NEDDS successfully. (K. Kohli, et al, 2010).

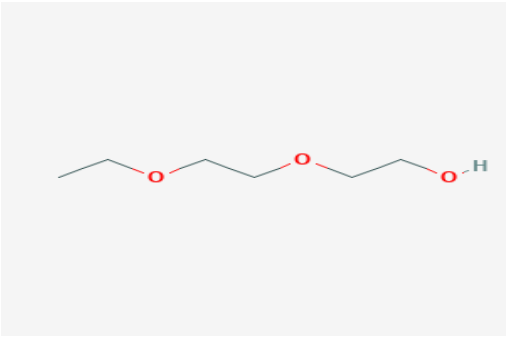
Cremophor RH 40 is a non-ionic emulsifier and solubilizing agent which is obtained by the reaction of ethylene oxide and hydrogenated castor oil. It conforms to the current standards of the European/US Pharmacopoeia. It is used for solubilizing aetherolea, aroma compounds, vitamins and hydrophobic APIs in aqueous-alcoholic and purely aqueous solutions. The finished formulations are especially stable. The main components of Cremophor RH 40 are fatty acid esters of polyethylene glycol and glycerol polyethylene glycol oxystearate.

The HLB value of Cremophor RH 40 ranges between 14 and 16. The particularly valuable features of this solubilizer are that it is almost odourless and—in aqueous solutions—nearly tasteless (WWW.CHEMIPAN.COM).



Formula 2.: Chemical structure of Cremophor RH 40.

Labrasol (CAPRYLCAPROYL MACROGOL GLYCERIDES) is a hydrophilic, water-dispersible surfactant used in SM/NEDDS for solubility and bioavailability enhancement. It is also used as a W/O surfactant for solubility and drug penetration enhancement in dermal delivery. It is a colourless liquid with a HLB value of 12 (www.gattefosse.com).



Formula 3.: Chemical structure of Labrasol.

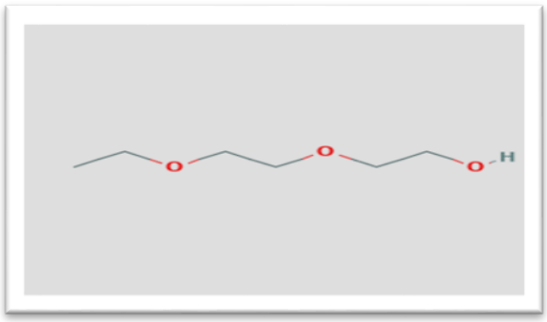
Surface active molecules may not sufficiently reduce the interfacial tension; therefore, it is necessary to add a **co-surfactant** or a co-solvent. With the help of a co-surfactant, the

surfactant can form very small droplets (diameter < 100 nm) in the dispersion. These agents are able to decrease the interfacial tension to a further extent, make the interfacial film less rigid, reduce the long range packing order of the surfactant molecules and optimize the interfacial curvature (Alany, R. et al., 2006).

Water soluble co-solvents/co-surfactants, such as ethanol, polyethylene glycol (PEG), propylene glycol and glycerol are commonly formulated into lipid based dosage forms. Their function is: (a) to increase the solvent capacity of formulation as it has the ability to dissolve considerable amounts of a drug and increase the solubility of the drugs that are freely soluble in them. However, there is a risk of drug precipitation when SM/NEDDS are dispersed in water because solvent capacity is lost upon dispersion. The co-solvent will separate from the hydrophobic phase and dissolve in the hydrophylic phase upon dispersion and, consequently, the drug will partially precipitate; (b) with the help of orally administrable organic solvents (Transcutol HP and polyethylene glycol), either the hydrophilic surfactant or the drug in the lipid base may dissolve extensively (Cui et al., 2009; Xie et al., 2009; Zhang et al., 2012) since SM/NEDDS require high concentrations of surfactants to ensure proper dispersion of the formulation, (c) to make the nanoemulsion more stable by wedging themselves between surfactant molecules (Benita, S., et al.2006).

There are several factors that have to be remarked before using a particular co-solvent. Co-solvents are miscible with oil only up to a specified limit. There are some incompatibilities of using alcohol and other volatile co-surfactants because these components evaporate into the shell of soft or hard gelatin capsules and as a result the drug precipitates. In addition, co-solvents can make the surfactant layer more flexible (co-surfactants) and facilitate the process of emulsification. On the other hand, if co-solvents diffuse away from the formulation, oil-water interface disruption can occur. There are some organic solvents which are suitable for oral administration, for example, in this research project, diethylene glycol monoethyl ether (DEGEE) is utilized as a co-solvent for all SM/NEDDS formulations (Gupta RN, et al,. 2009).

Transcutol HP (diethylene glycol monoethyl ether) is a powerful solvent for solutions, suspensions, SMEDDS and microemulsions. It is a topical penetration and permeation enhancer that has been used in capsules, injection and inhalation products. Molecular formula: C6H14O3 (www.gattefosse.com).



Formula 4.: Chemical structure of Transcutol HP.

Using lipid based formulations as carrier systems is a good option for those **active pharmaceutical ingredients** (API) that show poor solubility and stability which leads to insufficient absorption and decreased drug bioavailability (D. J. Hauss, 2007). The intended API must have appreciable solubility in oil phase in order to formulate the drug in SM/NEDDS (C. W. Pouton et al, 2008). When the dose of the API is very low, higher bioavailability would be gained from SM/NEDDS, especially for drugs with high octanol/water partition coefficient. Drug absorption from SM/NEDDS is essentially dependent on its solubility in water and oily phase (C. W. Pouton, 2000).

Table 2.: Chemical description of the excipients of SNEDDS components (Ujhelyi et al. 2012)

The name of SNEDDS components	**Chemical description**
Labrasol	Well-defined mixture of mono-, di- and triglycerides and mono-, di- fatty acid esters of polyethylene glycol. HLB = 14 Ester content 90% Lauric acid (C12) < 3 % Caprylic acid (C8) 50-80 % Capric acid (C10) 20-50 % Caproic acid (C6) < 2 % Myristic acid (C14) < 1 %
Cremophor RH 40/Kolliphor RH 40	PEG-40 Hydrogenated castor oil. HLB = 14-16
Transcutol HP	diethylene glycol monoethyl ether
Capryol 90	Propylene glycol monocaprylate Monoesters content 90 % (caprylic acid (C8)). HLB = 6

Lauroglycol FCC	Propylene glycol monolaurate Monoesters content 45-70 % Diester content 30-55% Lauricacid (C12) 95 % Caprylicacid (C8) <0.5 % Capricacid (C10) < 2 % Myristicacid (C14) <3 % Palmiticacid (C16) < 1 %. HLB = 4
Isopropyl-myristate	Propan-2-yl tetradecanoate, isopropyl alcohol (a derivative of propane) and myristic acid ester

5.3.Mechanism of self-emulsification

The process of self-emulsification is not well understood. Nevertheless, some author suggested that self-emulsification takes places when the entropy change favoring dispersion exceeds the energy required to increase the surface area of the dispersion (Vonderscher J & Meinzer A, 1994, Gershanik & Benita, 2000; Nazzal et al., 2002). Moreover, the free energy of a conventional emulsion formation is a direct function of the energy required to create a new surface between the two phases; it is defined by the equation below:

$$\Delta G = \sum \mathrm{N}\pi\mathrm{r}2\sigma$$

G represents the free energy associated with the process (ignoring the free energy of mixing), N is the number of droplets of radius r, and s is the interfacial energy.

As time passes, a tendency for separation of the two phases occurs in the emulsion which tries to reduce the interfacial area, and eventually, the free energy of the systems. Thus, for the stabilization of emulsions obtained by aqueous dilution, conventional emulsifyiers are used. These agents act as barrier that prevents coalescence and reduces the interfacial energy by forming a monolayer around the droplets in the emulsion. Emulsification occurs only if the interfacial structure has no resistance to surface shearing (Karim A et al., 1994).

5.3.1. SM/NEDDS preparation

The ability to keep the drug solubilized is a general requirement for all types of lipid formulations. By considering the amount of hydrophilic components, formulations can be divided into two sub-categories. Generally, high-energy methods using special equipment (e.g. ultrasound generators or high pressure homogenizers) are utilized for the formulation of self-nanoemulsions. These methods can provide sufficient energy to increase oil/water interfacial area in order to produce droplets of submicron size (Asua JM.et al, 2002). In the case of low-energy methods, formulation of nanoemulsions can be done by spontaneous emulsification which is independent of energy and device; the intrinsic properties of the components make it possible to produce submicron droplets. The process can be triggered by mixing of two phases—the lipophilic phase, which contains a dissolved hydrophilic surface active agent that forms a homogeneous liquid with the co-solvent and the API; and the hydrophilic phase, which is water. Upon contacting both phases, the hydrophilic surfactant in the oily phase is quickly dissolved into water by the amphiphiles. The weight ratio of oil/surfactant controls the droplet size of nanoemulsions (Anton N, et al, 2009).

Hoar and Schulman suggested a simple method for formulating microemulsions. When preparing an O/W self-microemulsion, where water is the continuous phase, we begin with a w/o emulsion using a low-HLB surfactant. An aqueous solution of a surfactant with high HLB value is added to this emulsion while stirring. When a certain amount of surfactant has been added, a ʻgelʼ phase is formed; subsequent addition of the surfactant solution will induce a phase inversion into an o/w type emulsion. The formulation becomes self-emulsifying by adding a hydrophilic surfactant (HLB>12) and/or a water soluble co-solvent to the triglyceride. The technique of formulation has importance in tackling poor hydrolytic stability and low drug solubility (Hellweg T, et al 2002). The co-solvent content of SMEDDS is high but they contain a lesser amount of oil. These types of formulations are exposed to the highest risk of precipitation (J.H.Schulman, et al 1959).

The terminology of the lipid-based systems (SMEDDS and SNEDDS) is very different in the literature. Generally, pharmaceutical scientist used SEDDS (self-emulsifying drug delivery system) as carrier system from SMEDDS to SNEDDS and the use of the terminiology does not only belong to the initial droplet size. According to Chatterjee et al. SNEDDS term should be used provided the emulsion droplet size is in the nanometer range (Chatterjee et al. 2016). Our composition containing antitumor agents the other compositions containing Plantago lanceolata extract belong to the category of SNEDDS.

5.3.2. Pseudoternary phase diagram

Pseudoternary phase diagrams are utilized to describe the phase behavior of different formulation components. Constructing a pseudoternary phase diagram is a necessary step for the preparation of a lipid-based formulation which self-disperses in the gastrointestinal tract as a thermodynamically stable nano drug carrier. Since the formation of nanoscale oil droplets requires very low free energy, the formulation develops in a thermodynamically spontaneous manner (Craig, D.Q.M., et al, 1995). The facilitation of self-emulsification is related to the facilitation of water penetration into the surfactant layer around the surface of the oil droplets (Rang, M.J., et al, 1999). A pseudoternary phase diagram was created to optimize the ratio of different components in a manner that the formulation obtained efficient self-emulsification potential (different zones of microemulsions) and it was optically isotropic and thermodynamically stable. In the diagram presented in Figure 4, each corner shows a binary mixture of two components, e.g. water/drug, oil/drug or surfactant/co-surfactant. It can be decided whether a region is a w/o or o/w microemulsion by simply considering if it is rich in oil or water (Shaji. J, et al, 2004).

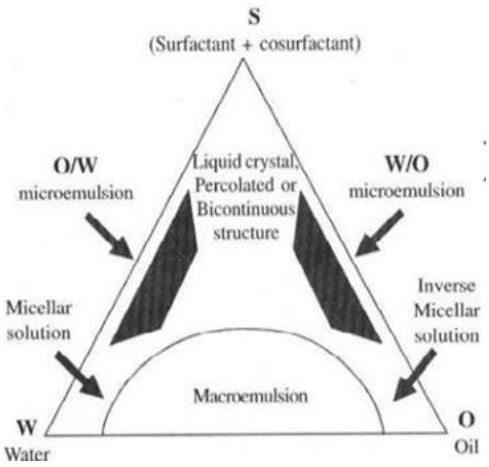


Figure 4: Hypothetical Phase region of Microemulsion system composed of oil, water and surfactant showing microemulsion region (Shaji. J, et al, 2004).

5.3.3. Advantages of SMEDDS over emulsions:

1. SMEDDS have the same advantages as emulsions that is they help solubilize hydrophobic drugs. Additionally, SMEDDS solve the problem of the layering of emulsions over time. Since SMEDDS belong to the thermodynamically stable systems, they can be stored easily (Constantinides, P.P., et al,1995) .

2. Microemulsions formed by the help of SMEDDS are thermodynamically stable and optically transparent. Droplet size represents the most important difference between the aforementioned microemulsions and common emulsions. The droplet size of common emulsions and SMEDDS-formed microemulsions normally ranges from 0.2 to 10 μm and from 2 to 100 nm, respectively (droplets of the latter size are called droplets of nano particles). As a result of the small particle size, there is a significantly larger total surface area for absorption and dispersion than in case of using solid dosage forms. Due to the easy penetration and absorption in the GI tract, the bioavailability of the drug improves (Peltola S, et al,2003).

3. While emulsions can be administered only as liquids (in case of internal applications), there are several delivery options for SMEDDS, such as filled hard gelatin capsules or soft gelatin capsules and they can be formulated even into tablets. (Kawakami K, et al, 2002).

SMEDDS offer following advantages:

- High drug payloads.
- Protection of sensitive APIs
- Formulation of microscopic droplets helps the drug distribute widely in the gastrointestinal tract and leave the stomach quickly.
- SMEDDS can decrease the irritation resulting from the prolonged exposure of the GIT to the drug.
- More consistent absorption-time profile
- Targeting the drug(s) selectively toward specific absorption windows in the gastrointestinal tract.
- Liquid or solid formulations.
- Incorporation of polymers in SMEDDS results in prolonged release of the drug.

- Easy manufacturing and scale up compared to other lipid formulations.
- Improved bioavailability due to increased solubility and better drug transport.
- Ability to deliver peptides that would otherwise suffer enzymatic hydrolysis in the gastrointestinal tract.
- Surfactants with high HLB values have been reported to increase drug permeability when administered along with the formulation as a result of the loosening effect on tight junctions.(Singh D,et al. 2014, Patel PA, et al, 2008 , Shukla P, et al, 2012).

Disadvantages of SMEDDS include:

- No reliable in vitro models are available for testing the formulations.
- As the basis of further development is in vitro – in vivo correlation, different lipid based formulations need to be developed as prototypes and examined in vivo in an appropriate animal model.
- Chemical instability and high concentrations of surface active agents in formulation cause irritation in the GIT.
- It is known that the volatile co-solvent in the conventional self emulsifying formulation migrates into the shells of hard gelatin capsules and as a result the lipophilic drug precipitates.
- Another problem concerning microemulsions is their unpleasant taste which is caused by the lipid content and results in reduced patient compliance; furthermore, due to the water content of microemulsions, soft or hard capsules cannot be used for encapsulation (Deokate UA, et al, 2013 . Shukla P,et al, 2012. Singh D, et al, 2014).

5.3.4. Role of SEDDS/SMEDDS in improving oral absorption

By using SM/NEDDS, the additional drug dissolution step preceding gastrointestinal absorption can be partially avoided. Increased drug absorption can be achieved as SM/NEDDS enhance drug solubilization in the intestinal fluids. In addition, another way to enhance absorption is using lipid based excipients in the formulation (A. S. Narang, et al, 2007).

5.3.5. Factors affecting SMEDDS

There are some main factors which can affect the efficacy of SMEDDS such as drug dose, drug solubility in oil phase, equilibrium solubility and the polarity of the lipid phase (Shukla P et al. 2012).

Nature and dose of the drug

Drugs administered in large doses are not acceptable and inappropriate for SM/NEDDS formulations except those with extremely good solubility in at least one SM/NEDDS component, ideally the lipophilic phase. Drugs with low oil and water solubility and with log P value about 2 are unsuitable candidates for SM/NEDDS (Constantinides PP.et al, 1995).

Drug solubility in oil phase

Drug solubility in the oil phase has a great effect on the ability of the SMEDDS to keep the drug in solution. When a drug is solubilized by the help of a surface active agent and a co-surfactant, diluting the SMEDDS can result in lowered solvent capacity of the surfactant and the co-surfactant and, consequently, the risk of precipitation increases (Shukla P, et al, 2012).

Equilibrium solubility

Potential precipitation in the GIT can be predicted by performing equilibrium solubility measurements. Nevertheless, in the GIT, crystallization can take place slowly because of the solubilising and colloidally stabilizing environment (Constantinides et al, 1995).

Polarity of the lipophilic phase

The polarity of the lipid phase is a governing factor in drug release from microemulsions. The polarity of the droplet is determined by the HLB, the molecular weight of the hydrophilic part, the concentration of the emulsifying agent and the chain and degree of unsaturated fatty acids. (Agrawal S, et al, 2012).

Visual assesment

Important information can be obtained about the status of self-emulsification and microemulsification in the mixture. Homogeneity, optical clarity, and fluidity can also be assessed. (Pujara ND et al, 2012).

5.4. Application and dosage form development of SMEDDS

Generally, oral intake is the most preferred drug administration route as it is painless and, compared to the parenteral route, the risk of infection is negligible which results in high patient compliance. On the other hand, the majority of *per os* administered drugs have low systemic availability and decreased efficacy due to instability. Increased thermodynamic activity of a drug in the vehicle results in higher absorption rates (Kawakami K, 2002). Self microemulsifying drug delivery systems are physically stable and easily manufacturable formulations for lipophilic drugs which show rate-limited absorption. These systems can improve the rate and extent of absorption and produce reproducible blood concentration-time profiles (Patel Jinalbahen Bipinkumar et al 2016). SMEDDS can effectively prevent four main drug degradation processes, such as hydrolysis, oxidation, photolysis and trace metal catalysis. However, in most cases, drug instability is caused by hydrolysis as those drugs have functional groups like ester, amide, lactone that may be susceptible to hydrolytic degradation. These sytems are considered to be optimal for drug delivery purposes due to these beneficial properties: low viscosity, protection against enzymatic degradation, easy preparation, surfactant provoked permeability, small droplet size (< 300 nm), improved drug stability and solubility (Mehta, S.K. et al. 2007).

Application of SMEDDS

- Loading the drug in SMEDDS improves solubility and bioavailability, as it circumvents the solubilization or dissolution step in the case of BCS II drugs such as ketoprofen (low solubility/high permeability) (Vergote GJ, et al, 2001,Sachan R,et al.2010).
- Protection of drugs from rapid biodegradation in the GIT caused by the low gastric pH, enzymes or hydrolysis. SMEDDS function as a barrier that separates the drug and the degrading environment (Sachan R,et al.2010).
- In Peptide drug delivery, the self-microemulsifying drug delivery system used for leuprorelin, a synthetic gonadotropin-releasing hormone (GnRH) demonstrated that SMEDDS can protect peptides from degradation by intestinal proteases (Fabian Hintzen,et al, 2014).
- SMEDDS for herbal drugs and traditional medicines: Numerous herbs and traditional medications are being used and applied for formulation and development of SMEDDS as most of them contain either volatile or fixed oil. Acteoside, an active constituent of herb extract has extremely low chemical stability in the presence of water due to its hydrophilic nature which leads to low oral bioavailability thus it causes hydrolysis upon reaction with water. By using SMEDDS formulations, bioavailability increased 4 folds (Prasad H, et al.2014).
- Improving activity of antitumor agents, reducing toxicity arising from these agents by reducing dose and keeping the drug in dissolved state (Thomas, N.et al, 2012).
- Improving stability of natural products (Zhang, L.; et al 2013).

5.4.1. Capsule filling with liquid self emulsifying system

Filling capsules is the easiest and the most frequently used method of encapsulating liquids or semisolid formulations for oral intake. In the case of liquid formulations, it includes a procedure composed of two-steps: filling the formulation into the capsules and right after that, sealing the cap and the body of the capsule, either by microspray sealing or by banding. Capsule filling is advantageous because it is a simple process, suitable for low-dose highly potent drugs and has high drug loading potential (up to 50% w/w) (Jannin V. et al. 2008).

Liquid S(M)EDDS encapsulated into soft or hard gelatin capsules are more acceptable as dosage forms. (McClintic JR et al,. 1976). Compared to conventional oral dosage forms, self emulsifying formulations are more effective in improving the rate and extent of absorption of lipophilic compounds (Charman SA et al, 1992).

5.5. Active pharmaceutical ingredients in SNEDDS formulations

5.5.1. Antitumor agents

Cervical cancer

Globally, cervical cancer—a largely preventable disease—is one of the most frequently occurring gynaecological malignancies. According to the National Cancer Institute, cervical cancer is the 3rd most frequently occurring cancer in women and the 2nd most common cancer-related death cause, resulting in almost 300,000 yearly deaths worldwide (Rong, C. et al,.2017, www.cancer.gov). Inflammation is one of sign of cancer and persistent inflammation is associated with malignant transformation, proliferation and metastasis. Inflammation causes changes in the tissues by the conversion of vascular, epithelial and immune cell functions. Growth factors, cytokines, chemokines, and lipid mediators are involved in the process of inflammation (S. Deivendran, et al, 2014). Inflammation and infections can promote and/or exacerbate malignancies.Malignant cells produce soluble mediators. These agents promote the progression of the tumor by recruiting and activating inflammatory cells. The following inflammatory mediators are known to have a role in inflammation-mediated cervical cancers: tumor necrosis factor-alfa (TNF-α), reactive oxygen species (ROS), interleukin-1 (IL-1), interleukin-6 (IL-6), interleukin-8 (IL-8), interleukin-18 (IL-18) and chemokines (Wan-wan Lin et al 2007). Physicochemical properties of antitumor agents can be seen in Table 2., in the end of this chapter.

Cisplatin

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. At room temperature, cisplatin is a white or deep yellow to yellow-orange crystalline powder. Cisplatin provides effective treatment for other types of malignancies as well, including sarcomas, carcinomas, lymphomas and germ cell tumors. Its mechanism of action relies on generating DNA crosslinks. Combination therapy that uses cisplatin along with

other medication is applied in the treatment of both the resistant and sensitive cell lines of ovarian cancer (T.Thigpen, et al 1979,Shaloam Dasari et al, 2014). The chemical structure of cisplatin is shown in figure 5 (www.enzolifesciences.com).

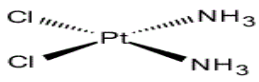


Figure 5: Chemical structure of Cisplatin.

Bleomycin

Bleomycin sulfate is a mixture of glycopeptide antitumor antibiotics produced by the bacterium Streptomyces verticillus. It has a unique mechanism of antitumor activity that is used to treat testicular cancer, lymphoma, cervical cancer. The drug binds to guanosine-cytosine-rich portions of DNA and inhibits DNA metabolism (medicines.org.uk, Dorr RT, 1992). Figure 6 presents the chemical structure of Bleomycin sulphate (www.enzolifesciences.com).

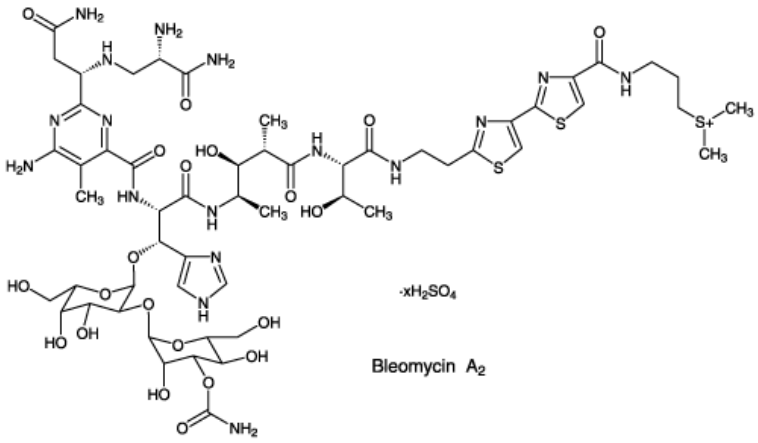


Figure 6: Structure of Bleomycin Sulfate

I-Phosphamide

Ifosfamide is an anti-cancer (antineoplastic) chemotherapy drug. It is widely used in the treatment of cervical, ovarian, testicular and head and neck malignancies along with soft tissue sarcomas and lymphomas. Ifosfamides are prodrugs of antitumor alkylating agents requiring hepatic P-450-catalyzed metabolism to become cytotoxic (You-Jung Shin et al, 2011,Sladek NE, 1988). The picture below is the chemical structure of Ifosfamide (www.selleckchem.com).

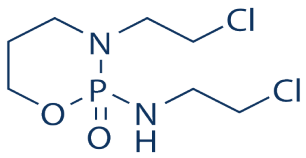


Figure 7: Ifosfamide structre.

Table 3. : Physicochemical properties of antitumor agents

	Solubility	**logP**	**pKa**	**Permeability**
Cisplatin	2.530 mg/mL	-2.19	strongest Basic 5.06	human intestinal absorption +/- Caco-2 -
Bleomycin sulfate	0.125 mg/mL	-0.52	strongest acidic: 11.32 strongest basic: 7.62	human intestinal absorption +/- Caco-2 -
Ifosfamide	3.78 mg/mL	0.86	strongest acidic: 13.24 strongest basic 0.12	human intestinal absorption +/- Caco-2 -

However, these components are hydrophilic, but their permeability is not so appropriate. That was the reason why we used SNEDDS compositions to enhance the penetration of these antitumor agents.

5.5.2. Plantago Lanceolata

The Plantaginaceae family contains more than 200 species worldwide. **Plantago lanceolata** is a perennial species with one to several rosettes (Goncalves 2016). This plant is safe to consume and historically, it has been widely used for medical purposes, such as the treatment of bleeding and tissue damage (Chevallier. A.1996). Plantago lanceolata also has antioxidant (Chiou 2004), anti-inflammatory (Lee 2005), antineoplastic (Xiong 1999), hepatoprotective (Yang 2006), immunoregulatory, neuroprotective, astringent, demulcent, mild expectorant and antibacterial properties (Chopra. R. N., et al 1986). 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 (He et al. 2011).

Botanical description of Plantago

Plantago lanceolata (Figure 8) (commonly known as ribwort plantain, English plantain, narrow-leaf plantain) is a species of flowering plants broadly distributed in the grassland of the temperate climate zone. (Goncalves, S.et al 2016) Flowering lasts from April to the first frost (Sagar and Harper 1964). Plantago lanceolata has rosette-forming, narrow, dark green, lance-shaped leaves and short, stubby, blackish flower head sitting on the top of a wiry stem (Michiko Murai et al,1995).

Figure 8:Plantago lanceolata (online Virtual Flora of Wisconsin. 2017).

Principal chemical components of Plantago:

Plantago lanceolata contains numerous biologically active compounds, many of which are present in large amounts.

- **Iridoid glycosides:**

The herbal substance contains approximately 2-3% iridoid glycosides. The main components are aucubin and catalpol accompanied by asperuloside, globularin and desacetylasperuloside-acid methylester. The maturity of the leaves affects the iridoid content, which can be as high as 9% in young leaves while the older ones contain iridoids only in traces. Generally, a drying temperature of 40-50°C is used for this herbal substance. Aucubin content decreses during drying. The effects of iridoid glucoside aucubin and its derivatives include antimicrobial (Rombouts & Links 1956; Ishiguro *et al.* 1982), laxative (Wagner & Wolff 1976); tissue

growth promoting and non-steroidal anti-inflammatory (Salas-Auvert *et al.* 1985); hepatoprotective (Yang et al. 1983; Chang *et al.* 1984); weak anti-oxidant (Toda *et al.* 1985); and uric acid excretion stimulating effects (Kato 1946).

- **Phenylethanoid glycosides**

The phenylpropanoid glycoside verbascoside (syn: acteoside) is also present in Plantago lanceolata. Verbacosid content can be as high as 9% of dry matter (Fajer et al. 1992). This compound also has biological effects, such as antimicrobial (Andary et al. 1982) and antifungal effects (Shoyama et al. 1986), in-vitro antitumour activity (Pettit et al. 1990; Herbert et al. 1991); it is a strong superoxide anion scavenger and antioxidant (Zhou & Zheng 1991) and has anti hypertensive and anti-tremor activity (Andary et al. 1982).

- **Mucilage:**

The genus Plantago is a well known source of mucilage or polysaccharide hydrocolloids (Morton 1977; Franz 1989). The mucilage content of plantain leaves is about 0.8% (Brautigam & Franz 1985). Mucilage forms a viscous gel with water by slow hydration, which causes laxative and purgative effects in the intestines (Duke 1992). Plantago mucilage is used in veterinary products to control bovine diarrhoea (Verschoor 1987).

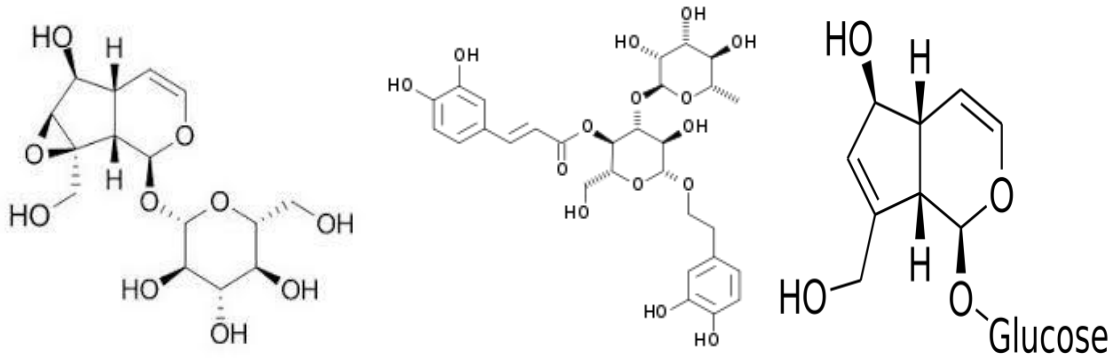
The herbal substance also contains 6.5% tannins, phenolic carboxylic acids, including phydroxybenzoic-, protocatechuic, gentisinic-, chlorogenic- and neochlorogenic acid, among others. The coumarin aesculetin, the xanthophyll decomposition product loliolide and small amounts of a hemolytic and antimicrobial saponin are also present, as well as volatile oil. The high sorbitol content of the plant (generally about 2% but can be higher during periods of drought and under saline conditions) can be exploited in taste masking. This sugar alcohol has 60-70% the sweetness of sucrose (Oku 1992) and—along with mannitol—funtions as the main osmoregulator in Plantago lanceolata, which differs from other grasses, in which this function is fulfilled by the amino acid proline. Flavonoids, mainly apigenin and luteolin are also present in plantain (Lewis 1984).

- **Mineral composition**

Plantain has high nutritional value and contains considerable amounts of calcium, magnesium, sodium, phosphorus, zinc, copper, cobalt, iron and vitamins A, C, and K (J.L. Guil Guerrero,2010).

- **Chemical structures of bioactive components in plantain (Plantago lanceolata)**

Phenylethanoid glycosides are important metabolites in Plantago species. They are derivatives of benzoic acid containing a phenylethyl ring linked to β-glycopyranose via an ester or glycosidic bond. Verbascoside (acteoside) and plantamajoside are the most remarkable representatives of this group (Rønsted et al., 2003). Phenylethanoid glycosides and iridoid glycosides protect the Plantago genus against pathogens and herbivores and mediate plant–plant interactions functioning as allelopathic signals (Pankoke et al., 2013). Figure 9 presents the chemical structures of different bioactive components in Plantago lanceolata (Chemfaces.com,Wu YT et al, 2012,Alcibiades 2007).



Catalpol (CA) **Acteoside/Verbescoside (ACTE)** **Aucubin (AU)**

Figure 9: Structures of bioactive components in Plantain (Chemfaces.com,Wu YT et al, 2012,Alcibiades 2007).

Medical use of Plantago lanceolata

The role of Plantago species in traditional medicine provided inspiration for the evaluation of numerous pharmacological extracts for their potential antioxidant, anti-inflammatory, antibacterial antiviral, hepatoprotective, antineoplastic, immunostimulant (antibody production) and wound healing properties among others. Some prominent pharmacological studies are outlined in the following section. (Chiou, W.F.;et al,.2004,Lee, J.Y.;et al,.2005, Xiong, L.;et al,.2016).

Anti-inflammatory and antioxidant effects

The modified hen's egg chorioallantoic membrane test (HET-CAM) has been used to evaluate the anti-inflammatory effect of Plantago lanceolata (Marchesan *et al.* 1998). The effects of

Plantago lanceolata extracts (leaves, flowers, roots) on mediators of inflammatory response have been studied *in vitro* in murine macrophages (Vigo *et al.* 2005). These extracts inhibited nitric oxide production in this cell line and effectively scavenged nitric oxide radicals. The anti-inflammatory effects of Plantago lanceolata extracts are results of nitric oxide inhibition not of the reduction of prostaglandin production. The phenylethanoid acteoside and the iridoidglycosides catalpol and aucubin (Recio *et al.* 1994) expressed antiinflammatory effects (*in vitro* and *in vivo* studies). The antioxidant capacity of Plantago lanceolata has also been investigated in relation to its anti-inflammatory effects as free radicals are potential factors in inflammatory diseases. In a study, Herold et al.(2003) examined the mechanism of the antioxidant effects of a Plantago lanceolata leaf tincture standardised to mucilaginous substances. The study included a colorimetric assay for measuring the antioxidant activity; the free radical scavenging potential was investigated by means of activated human polymorphonuclear neutrophils (PMNs). The ticture was found to have a slight antioxidant effect and capability of scavenging free radicals released by activated PMNs. Single compounds like acteoside have also been proven to have antioxidant properties (Ji et al. 1993, Pan and Hori 1996, Wang et al. 1996; Li et al. 1996, Hausmann et al. 2007)

Antibacterial effects

The iridoid glycosides aucubin and catalpol and the phenylpropanoid glycoside, acteoside (synonym: verbascoside) are the best-known biologically active compounds in Plantago lanceolata (Stewart, 1996; Tamura and Nishibe, 2002). They were proven to be antifungal and antimicrobial compounds (Andary et al., 1982; Davini et al., 1986; Kim et al., 2000).

Traditional use of Plantago lanceolata:

Current uses of P. lanceolata include the treatment of oral, pharyngeal and upper respiratory illnesses and, when topically applied, cutaneous conditions. In case of injury, it can be appled directly on the wound; it helps stop bleeding, promotes healing and alleviates pain. It also eases itching. Plantago lanceolata is considered useful in the tratment of snake and insect bites and nettle stings. Additionally, it has been used in the treatment of dermatitis, psoriasis and superficial burns. Plantago lanceolata reduces airway mucus hypersecretion which is beneficial in the treatment of common cold, catarrhal inflammation, sinus infection and pulmonary and bronchial allergies such as allergic rhinitis and asthma. As this herb also has

astringent properties, it has been effectively used in the treatment of diarrhea, gastritis, colitis and other digestive disorders (Oloumi et al., 2011).

Pharmaceutical dosage forms of Plantago lanceolata

There are several different pharmaceutical products of Plantain which are marketed for internal and external applications (primarily in Europe). Plantago lanceolata is available as the following dosage formulations: effervescent tablet, syrup, lozenge, oral liquid, coated tablet and herbal tea. Pharmacopoeias of various countries list P. lanceolata as a safe herb (Blumenthal, 1998). Generally, the physicochemical properties of natural products lead to chemical instability and low in vivo bioavailability. The formulation may result in increased solubility, better stability and higher biocompatibility. Phenylpropanoid glycosides are widely distributed in different plant families**.** As verbascoside is a highly hydrophilic compound, its application in drug formulations is limited. Verbacoside has been reported to be more stable in O/W emulsions (Vertuani S., 2011). SMEDDS were brought into focus as formulations that can stabilize herbal drugs and increase their bioavailability (Zhang et al. 2013). Micro- and nano-sized drug delivery systems carrying herbal drugs have been regarded as carrier systems ideal for optimizing the efficacy/activity of such extracts and for managing the difficulties associated with herbalism (Ma et al., 2012).

5.5. Biocompatibility investigations

One of the essential factors in every stage of drug formulation is drug safety profile. Biocompatibility of a material is the extent to which the it lacks injurious and/or toxic effects on biological systems. Based on the aspect that all compounds can be poisonous depending on the dose applied, toxicity testing is a necessary step in the early stage of drug formulation to determine safety thresholds not exclusively for the API but for every incorporated excipient as well (Rozman K, et al 2001). Excipients are included in drug formulations to aid manufacture, administration or absorption and must be able to aid the API in fulfilling its functions, that is, dosage, stability, and release of API from the formulation. Moreover, many excipients are used as penetration enhancers as they effect on biological membranes. This can be an advantageous property of excipients that is considered before the formulation but toxicity aspects should be discussed as well (Xiang Zhang, et al 2016). Many methods of toxicity analysis can be used to determine whether a chemical is hazardous or to rule out its

toxicity in the early phase of drug development. Early identification of toxic properties can save both time and expenses, and most importantly, reduce the risk of failure in a later phase of the development (S.Parasuraman, 2011, Albert P. Li, 2005). The results of cytotoxicity evaluation on different cell line models may be predict human specific toxicity. Cell-based assays are currently considered central to toxicity testing and are used for examining different molecules to determine whether the test materials affect cell proliferation or have direct cytotoxic effects that subsequently result in cell death (Terry L Riss,2016). It has been attempted to examine the toxic properties of pharmaceutical excipients and SMEDDS formulations using *in vitro* cell models. As reproducing the dynamics and the environment of the gastrointestinal tract is not possible by this *in vitro* model, it may have advantages in early phase toxicity testing. As surface active agents are required in high quantities to form microemulsions, the toxicity of surfactants should also be taken into account (Palamakula , A et al, 2004).

To obtain successful results in toxicology evaluation methods, comprehensive and tiered screening methods must be employed (Olson H, 2000). The biocompatibility of SNEDDS compositions containing antitumor agents and the biocompatibility of SNEDDS-PL compositions were tested.

5.5.1. Cell culture models

Cell culture defines as a method for growing eukaryotic cells under specific laboratory conditions including temperature, composition of gases, the growth medium and the substrate supporting the cells (Adamson et al, 2012). Cell culture assays are suitable means of investigating the biocompatibility of materials or extracts by utilizing isolated cells *in vitro*. Primary cells are isolated directly from living animal or human tissues and processed to establish them under controlled culture conditions. A cell line is defined as a cell culture that is formed from a single cell and possesses uniform genetic composition. The use of cell lines in cell culture-based research is an important element of all biomedical research. Selecting a specific cell line is an important issue since the compound activity may be specific (Ghanemi,A 2014, M.Yakut Ali et al, 2015). Cell lines can be finite or continuous. Virtually indefinitely proliferating cell lines are cost-effective tools in fundamental research.

In biomedical research, cell lines have a variety of applications including measuring the effects of radiation, drugs, chemicals, toxins and are also applied in the development of vaccines and pharmaceuticals. In tumor pharmacogenomic modeling, human cell lines have

been broadly utilized for predicting clinical response. The role of cancer cell lines in cancer research as experimental model systems is especially important (Niu N, et al, 2015). The major advantage of using cell lines—a batch of clonal cells—for any applications including screening of anticancerous drugs, determining cytotoxicity, *in vitro* screening of several drugs, etc. (Uppangala,2010) is that it produces consistent and reproducible results. *In vitro* models for different human cancers consist of primary cells or tissue explants obtained straight from surgical patient material or immortalized (continous) human cell lines with indefinite proliferative potential. There are several types of immortalized cell lines with various properties. They are categorized mostly on the basis of the cell type they originated from. For example, HeLa cells were isolated from a naturally occurring cervical cancer; Caco-2 cell line is an immortalized culture of heterogeneous human epithelial colorectal adenocarcinoma cells (Arun Malik et al 2013,Rahbari R, et al 2009). HeLa cells were used for the investigations of SMEDDS-BIP combinations and Caco-2 cell line was used for the testing of the cytotoxicity of SNEDDSP-L compositions.

Caco-2 cells

Several in vitro and in situ methods have been developed to evaluate and predict the absorption and permeation properties at the different drug administration sites of the human body and to study the underlying processes (Avdeef 2003).

Caco-2 (**Ca**ncer **co**li-2) cells are immortalized lines of heterogenous human epithelial colorectal adenocarcinoma cells isolated from human colon and when grown under specific cell culture conditions, they become differentiated and polarized like small intestinal cells. Caco-2 cells were established by Jorgen Fogh at the Sloan-Kettering Cancer Research Institute (Fogh et al. 1977). This cell line has been utilized broadly for modeling the human small intestinal mucosa and epithelial barrier to study the toxic properties of *per os* administered drugs and estimate their absorption. Caco-2 cells are a promising and advantageous means of modelling permeability in pharmaceutical experiments. (Artursson, P. 1991). Due to its common characteristics with the human small intestinal epithelium, like cell morphology, polarity, and enterocytic differentiation, it has been suggested that Caco-2 cell line is a suitable *in vitro* model system to predict the oral absorption of drugs in humans. Several experiments have proved that Caco-2 permeability coefficients are strongly correlated with absorption data in humans, especially if the mechanism of transportation is passive paracellular transport (Artursson and Karlsson 1991). A very useful property of Caco-2 cells is their uniqueness as they have the ability to differentiate spontaneously into one-cell-thick monolayers when reaching confluence, and as a result, they acquire properties typical of absorptive enterocytes with brush border layer, resembling the mature small intestine. In the pharmaceutical industry, this cell line is an advantageous and rapid means of in vitro screening in the field of drug discovery (Praveen V. Balimane et al, 2005, Sun et al, 2008). The pictures below represent electron microscope images of Caco-2 cell monolayers (www.lgcstandards-atcc.org/, HTB=37).

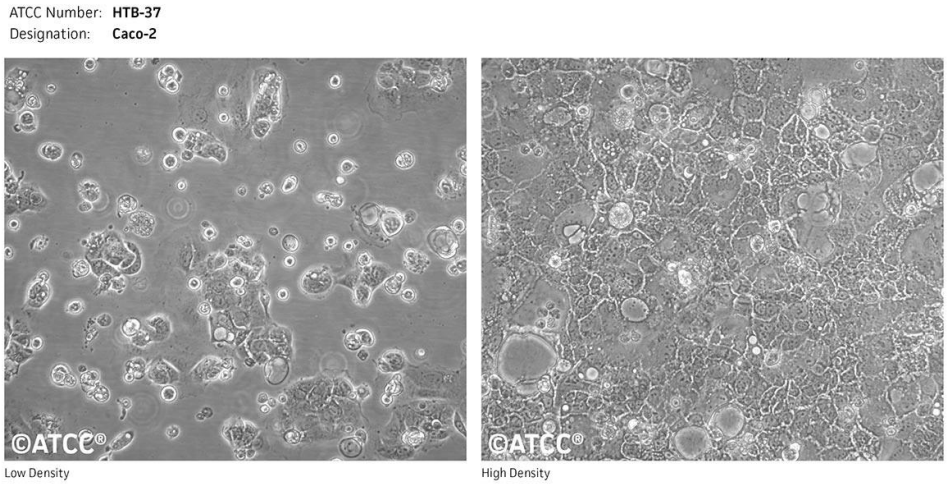


Figure 10: Caco-2 cells under electron microscope.

Characteristics of Caco-2 cells:

Caco-2 monolayer spontaneously differentiates and acquires functional and morphological (polarized columnar cells) properties of mature small-intestinal enterocytes. This cell line expresses different drug metabolizing enzymes, for example aminopeptidase, esterase and sulfatase. Essentials of Caco-2 culture media compositions are Dulbecco's Modified Eagle Medium (DMEM) with l-glutamine, 4500mg/1; D-glucose, without sodium pyruvate, 10%; HIFBS (Heat Inactivated Fetal Bovine Serum, inactivation at +56°C for 30 min with NaHCO3), 10% (V/V); nonessential amino acids (NEAA), 1% (V/V); L-glutamine, 0.876g/1; Penicillin, 100mg/ml; Streptomycin, 100 mg/ml. The unique properties of the Caco-2 cell monolayer are given in Table 4 (Hidalgo IJ;et al 1989).

Growth	Grows in culture as an adherent monolayer of epithelial cells
Differentiation	Takes 14–21 days after confluence under standard culture conditions
Cell morphology	Polarized cells with tight junctions and brush border at the apical side
Electrical parameters	High electrical resistance
Digestive enzymes	Expresses typical digestive enzymes, membrane peptidases and disaccharidases of the small intestine (lactase, aminopeptidase N, sucrase-isomaltase and dipeptidylpeptidase IV)
Active transport	Amino acids, sugars, vitamins, hormones
Membrane ionic transport	Na^+/K^+ ATPase, H^+/K^+ ATPase, Na^+/H^+ exchange, $Na^+/K^+/Cl^-$ cotransport, apical Cl^- channels
Membrane non-ionic transporters	Permeability glycoprotein (P-gp, multidrug resistance protein), multidrug resistance-associated protein, lung cancer-associated resistance protein

Table 4: Physiological characterization of Caco-2 cells (Hidalgo IJ;et al 1989).

Hela cells

In vitro cell culture models are utilized in the evaluation of the antitumorigenic activity of new candidate medications. Biomedical scientists utilize human cells grown *in vitro* to study cell functions and test hypotheses on the causes and therapy of different conditions. The cells should be "immortal"—that is, be able to grow indefinitely, survive long periods of freezing; also, it shoud be possible to divide them into different batches and share them among researchers. HeLa cells were the first immortal human cells that could survive *in vitro* (in a

test tube) (Niu N, et al, 2015). The code HeLa consists of the first two letters of a cancer patient's given and family name. The cell line originates from the cervical cancer cells taken from Henrietta Lacks (who died of the disease) in 1951 by the cell biologist George Gey (Scherer WF et al,1953). After more than six decades, there are uncountable HeLa cells in research facilities across the globe; it is the most commonly used cell line in laboratories. Science reached several milestones—e.g. gene mapping, cloning, in vitro fertilization—using Henrietta Lacks' cells which became the best-known continous cell line. (Jones et al., 1971).

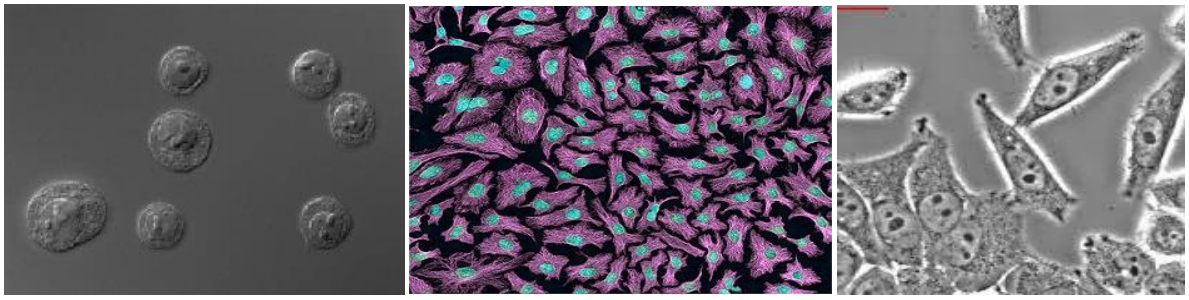


Figure 11: Scanning of electron and confocal laser microscopic pictures of Hela cell line.

HeLa cell line characteristics:

The size of a HeLa cell is about 20 microns in diameter. They are adherent immortal cells isolated from cervical tissue (John R. Masters, 2002).

Scientific application of Hela cells:

HeLa cells are used for transient transfection, gene mapping, HIV/AIDS and cancer research including studying the effects of radiation and toxic substances, defining cancer markers in RNA and investigating fullerenes to induce apoptosis as a part of photodynamic therapy (Smith, Van ,2002, Scherer, W.F 1953).

5.5.2.Cytotoxicity investigations

Cell cytotoxicity is the ability of certain chemicals or mediator cells to damage living cells. In drug research, cytotoxicity assays are widely utilized for screening cytotoxic effects of compounds. Using human cells is an essential modelling step in early toxicity investigations (Albert P. Li,2005). Exposing cells to cytotoxic compounds results in different types of cell deaths as either necrosis (cells lose membrane integrity and die rapidly as a result of cell lysis)

or apoptosis (cytoplasmic shrinkage and nuclear condensation) can occur (Promega, 2012). Different reagents can be utilized for detecting cell viability; they can indicate various cell functions like co-enzyme production, enzyme activity, cell adherence, cell membrane permeability, nucleotide uptake activity and ATP production (Riss T.L. et al, 2004). Different methods have been established to determine the number of living cells, such as tritium-labeled thymidine uptake method, crystal violet method, colony formation method, MTT and WST methods (Shilo M. Smith et al, 2011).

MTT cell viability assay:

MTT assay is a colorimetric method which is used for the assessment of cell metabolism indicated by the reduction of MTT dye to Formazan dye. Provided certain conditions are met, NAD(P)H-dependent cellular oxidoreductase enzymes may indicate the amount of living cells. MTT is a pale yellow tetrazolium dye that gets reduced only by living, metabolically active cells (primarily by mitochondrial dehydrogenases) and in this case, it turns into an insoluble, purple formazan dye (T.Mosmann et al.1983). As the dye reduction is proportional to the number of living cells, measuring the optical density (OD) of the purple formazan product provides an estimate of cell viability. This method is widely used for assessing the effects of nanoparticles on cell viability (M.M. Song,et al. 2010).

The main applications of MTT assay are evaluation of viability (cell counting), cytotoxicity and cell proliferation (cell culture assays). MTT reduction is specifically carried out by oxidoreductase enzymes in the mitochondria, as well as by endosome and lysosome compartments (M. V. Berridge et al. 2005). Figures 12 and 13 present a microplate after an MTT assay and chemical structures of MTT, respectively (Terry L Riss, et al, 2016).

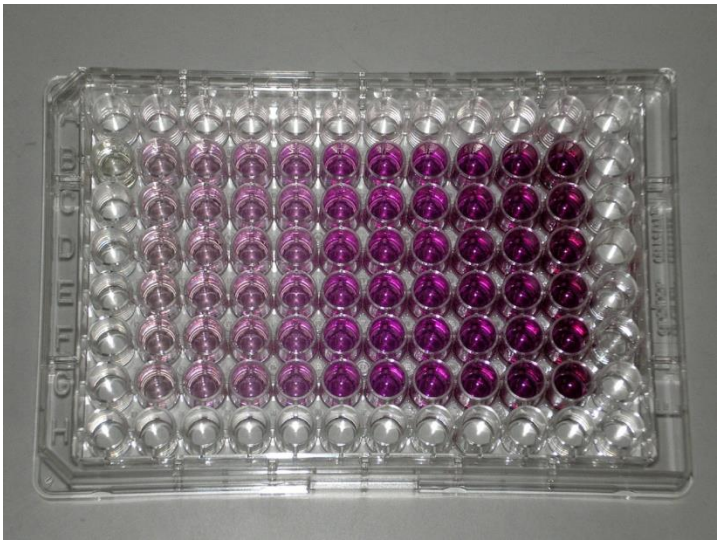


Figure 12: The result of MTT-test on Caco-2 cells

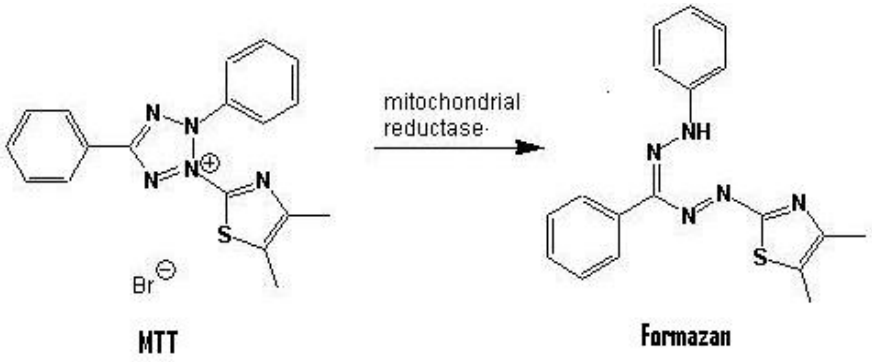


Figure 13: Structures of MTT and colored formazan product (Terry L Riss, et al, 2016).

Cell viability assay calculates by using below equation: (Meng Xie et al, 2016)

$$Cell\ viability\ (\%) = \frac{\mathbf{OD\ (test) - OD(blank)}}{\mathbf{OD(negative\ control) - OD(blank)}} * \mathbf{100\%}$$

We used MTT-tests for both compositions.

Inhibition of Cell proliferation

Cell proliferation tests are essential parts of cell growth and differentiation experiments and are frequently used for the evaluation of both chemical toxicity and inhibition of tumor cell growth in drug development. These assays provide information on the number of cells or cell divisions, metabolic activity or DNA synthesis over a period. Both the rate of proliferation and the ratio of living cells can be estimated by cell counting methods which use viability indicating dyes. The inhibitory effects of antitumor agents can be evaluated by Cell proliferation assays. The MTT in *vitro cell* proliferation assay is a broadly utilized method for assessing antitumor activity of both synthetic and natural compounds. This assay evaluates the inhibitory effect of antitumor agents which can be given as IC50 (50% inhibitory concentration) values (MxCauley et al, 2013). This method was used for the testing of SMEDDS-BIP compositions.

5.6. Investigation of drug action in formulations

As SMEDDS are pre-mixtures of a drug, oil, surface active agents and co-surfactants, increased bioavailability of oil-based drugs delivered in the form of microemulsions is reported by several studies. Moreover, the rate of absorption can be increased by reducing particle size. Various assays are available for evaluating the activity and efficacy of APIs in SM/NEDDS formulations (Ajeet K. Singh et al 2009)**.** To assess the antioxidant potential of the API in SNEDDS, different *in vitro* systems can be used, e.g. DPPH, ABTS, FRAP, $OH^{\bullet}$, β-carotene (Kandhasamy Sowndhararagan et al, 2013). Mouse ear edema test is a suitable assay for investigating the anti-inflammatory property of API-containing SNEDDS (Chu-Wen Li et al.2013).

5.6.1. DPPH test

Polyphenolic compounds are common in plants; they exert various biological effects such as antioxidant activity (Zubeyir Huyut, et al,2017). Antioxidant compounds in food and medicinal plants have attracted interest over the past few decades due to their ability to either delay or inhibit oxidation caused by atmospheric oxygen or reactive oxygen species. Different analytical methods are available to evaluate antioxidant capacity, including DPPH, ABTS, FRAP, etc. (Aurelia Magdalena Pisoschi et al, 2011).

The stable free radical 2,2-diphenyl-1-picrylhydrazyl is commonly abbreviated as DPPH. This organic compound is broadly utilized in biochemistry to assess the free radical scavenging activities of plants. Due to the delocalization of an electron in the DPPH molecule, it has purple color and shows a strong absorption at 520 nm. The spectrophotometric method measures the change in the concentration of DPPH upon reaction with antioxidants. Thus, antioxidant activity can be evaluated on the basis of the decreased absorption at 520 nm (yellow color) (Aurelia Magdalena Pisoschi et al, 2011, Krystyna Pyrzynska, et al, 2013). Figure 14 shows the principle of the DPPH radical scavenging capacity assay (Krystyna Pyrzynska et al 2013).

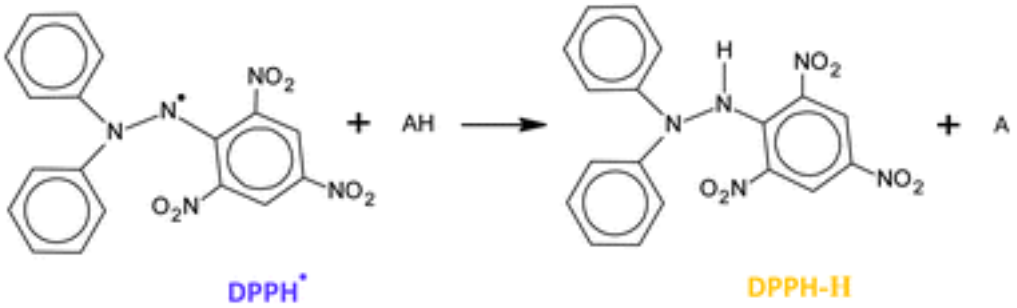


Figure 14:The addition of sample compound to the DPPH• may change its color from violet to yellow.

DPPH assay is rapid, simple and inexpensive compared to other methods. The scavenging effect can be calculated as follows: (Cuiqin Li et al, 2017).

$$\%\,\mathrm{DPPH}^{\cdot} = E\% = \left(\frac{A_0 - A_t}{A_0}\right) \times 100$$

A_0 and A_t are the absorbance of the mixture/solution at 520 nm at time 0 and time t, respectively. This method was used for testing the antioxidant effect of SNEDDS-PL compositions.

5.6.2. Ear edema test

Ear oedema models in mice are sensitive and reliable tests for anti-inflammatory activity of orally and topically applied agents. Ear oedema can be induced by applying xylene (dimethylbenzene) or any other inflammation stimulators to the anterior or posterior surface of the ear (conventionally the right ear) of a rat/mouse and the resulting irritant effect can be used for testing anti-inflammatory activity. Measuring the thickness of the inflamed ear before and after the treatment and comparing it to a control (left ear) are essential steps of this method. The anti-inflammatory and anti-edema properties of an API can be evaluated by this assay on the basis of the reduction of ear thickness (Wa Mulla et al. 2010). This method was used for testing the antiinflammatory effect of SNEDDS-PL compositions.

6. THESIS OBJECTIVES

The main purpose of this research is to prepare SNEDDS 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 SNEDDS carrier on human cervical cancer HeLa cells. Our experimental design can be seen in Figure 15.

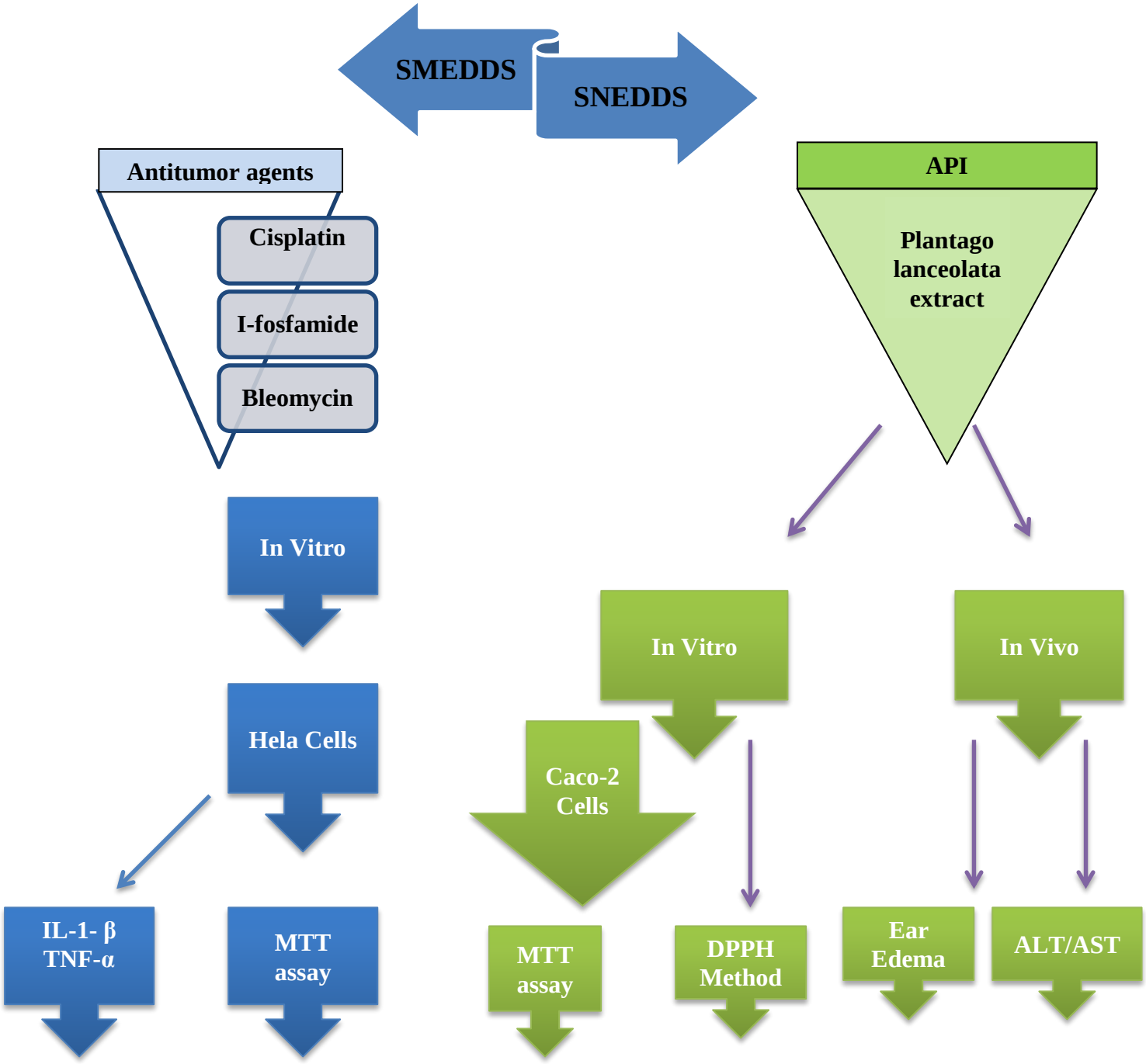
The specific objectives of the thesis were to:

- 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 SNEDDS upon dilution with water and evaluate the droplet size controlled by Dynamic Light Scattering Method (DLS)
- Evaluate the biocompatibility and toxicological profile of SNEDDS 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 SNEDDS 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.

Figure 15: Experimental design of the thesis



7. EXPERIMENTAL PART

7.1. Materials

7.1.1. SNEDDS components

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.

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).

7.1.2. Active pharmaceutical ingredients in the compositions of different SNEDDS

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).

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 (100 g Dry Weight DW—400 mL MeOH) for 30 minutes followed by filtration process. Rotary evaporator used to dehumidify the powder into completely dry form. Afterwards the herb extract was defatted with hexane (3 × 50 mL) and dried again. 25.2 g dry extract yielded from 100 g of herbal

drug. After dissolving dry extract with MeOH 10mg/mL solutions were prepared and diluted 100 to 250-fold for dissociation of sample. Accurate standards of bioactive components of Catalpol, Aucubin and Acteoside were used as standards to develop the calibration curves in the range of concentration 0.2-20 μg mL^{-1} (MeOH). 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 (column: kinetex XB-C_{18} 100 mm × 2.1 mm × 2.6 μm). Gradient components were A, water with 0.1% (*v*/*v*) formic acid; B, acetonitrile with 0.1% (*v*/*v*) formic acid. The time programme was 5% B: 0–1 min, 5–20% B: 1–5 min, 20–60% B: 5-9 min, 60–100%: 9–11 min; 100% B: 11–13 min; 100–5% B: 13–15 min, 5% B: 15–17.5 min. Flow rate was 250 μL/min. Injection volume was 1 μL. In positive mode iridoids were figured out and in negative ion mode acteoside was detected. Electrospray ionisation (ESI) parameters were including: heater temperature, unheated; sheath gas, N_2; flow rate, 8 arbitrary units (arb); aux gas flow rate, 0 arb; spray voltage, 5 kV; capillary temperature, 275 °C. Capillary voltage was 7 V and −35 V in positive ion mode and negative ion mode, respectively. Calibration curves of 0.2–20 μg mL^{-1} were used, and pure compounds were dissolved in MeOH.

7.1.3. Cell culture models

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 (Bigansoli E, et al, 1999).

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 (Palamakula, A.et al, 2004).

7.1.4. 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 10^4 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.

Inflammatory mediators

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

7.2. Methods

7.2.1. SEDDS preparations

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

Water and oil dilution method had been applied for preparation of 6 different SNEDDS formulations in combination with different surfactants and co-surfactants that their compatibility and solubility were screened formerly (Ujhelyi, Z et al, 2012). Table 5 depicted the 6 SNEDDS compositions. 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 chemotherpeutic 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. The basis of the selection of surfactant and co-surfactant was our previous work (Ujhelyi et al, 2012., Ujhelyi et al. 2013). MTT and LDH cytotoxicity tests and transport studies were made by different surfactants and co-surfactants. We selected those compounds which did not show high cytotoxicity and can increase the permeability of model drug.

According to the result of DLS test, we definite our compositions as SNEDDS, despite of the fact that the terminology of SMEDDS/SNEEDS is really debatable (Chatterjee et al, 2016).

Composi-tion	API (mg/ml)		Formulated self-emulsifying system components (ml)					
			Kolliphor RH40	Labrasol	Capryol 90	Lauroglycol FCC	Transcutol HP	Isopropyl myristate
1	0.001 - 0.05	Cisplatin	3	3	1,5	0	3	1,5
	0.1- 5.00	Ifosfamide	3	3	1,5	0	3	1,5
	0.0005 - 0,05	Bleomycin sulfate	3	3	1,5	0	3	1,5
2	0.001 - 0.05	Cisplatin	2,4	2,4	1,2	0	4,8	1,2
	0.1- 5.00	Ifosfamide	2,4	2,4	1,2	0	4,8	1,2
	0.0005 - 0,05	Bleomycin sulfate	2,4	2,4	1,2	0	4,8	1,2
3	0.001 - 0.05	Cisplatin	2	2	1	0	6	1
	0.1- 5.00	Ifosfamide	2	2	1	0	6	1
	0.0005 - 0,05	Bleomycin sulfate	2	2	1	0	6	1
4	0.001 - 0.05	Cisplatin	2,4	2,4	0	2,4	2,4	2,4
	0.1- 5.00	Ifosfamide	2,4	2,4	0	2,4	2,4	2,4
	0.0005 - 0,05	Bleomycin sulfate	2,4	2,4	0	2,4	2,4	2,4
5	0.001 - 0.05	Cisplatin	2	2	0	2	4	2
	0.1- 5.00	Ifosfamide	2	2	0	2	4	2
	0.0005 - 0,05	Bleomycin sulfate	2	2	0	2	4	2
6	0.001 - 0.05	Cisplatin	1,5	1,5	0	1,5	6	1,5
	0.1- 5.00	Ifosfamide	1,5	1,5	0	1,5	6	1,5
	0.0005 - 0,05	Bleomycin sulfate	1,5	1,5	0	1,5	6	1,5

Table 5 . SNEDDS compositions containing BIP combinations

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 (Ujhelyi, Z et al, 2012). The compositions are presented in Table 6. 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.

Compositions	Isopropyl-myristate	Transcutol HP	Kolliphor RH 40	Labrasol
1.	33 %	33 %	33 %	-
2.	25 %	50 %	25 %	-
3.	15 %	60 %	15 %	-
4.	10 %	80 %	10 %	-
5.	33 %	33 %	-	33 %
6.	25 %	50 %	-	25 %
7.	15 %	60 %	-	15 %
8.	10 %	80 %	-	10 %

Table 6: Compositions of Plantago lanceolata extract (PL-SNEDDS) (1–8). The components were Isopropyl myristate as oily phase, Labrasol or Kolliphor RH 40 as surfactant, and Transcutol HP as co-tenside. The concentration of Plantago lanceolata extract was 10 mg/mL in all samples.

7.2.2. Investigation of SNEDDS compositions

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 (Malvern, Worchetershire, UK, 2000). Diameters of dispersed droplets according to the diffusion coefficient have been evaluated automatically by the computer program (Urban, C. et al, 1998). 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 (Malvern, Worchetershire, UK). Measurements were performed in quadruplets to obtain an average and standard deviation of the results.

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 μm 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.

7.2.3. Biocompatibility evaluations on different cell lines

MTT cell viability assay on Hela cell line with SNEDDS compositions containing antitumor agents

To exclude any toxic effect of the blank SMEDDS and their components on HeLa cells, MTT cell viability test was used (Palamakula, A. et al, 2004). 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 CO_2 incubator at 37 °C for 4 days. For these studies, the culture medium was removed, surfactant or SMEDDS 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 (Gursoy, N. et al, 2003).

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 (Gursoy, N. et al, 2003). 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 (Mensor, L.L et al, 2001).

7.2.4. Investigation of the activity of SMEDDS/SNEDDS compositions

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 10^4 cells per well in 100 mL of medium, and incubated for 3–4 h. Cytostatic drugs alone in cell culture medium or incorporating in SNEDDS 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% CO_2 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 ≤10%.

Inhibitory Effect of Different SMEDDS 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 10^4 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, the previously described HeLa cell proliferation test was used.

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 λ = 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. (Mensor, L et al, 2001).

$$\mathbf{AA\% = 100 - [((Abs_{sample} - Abs_{blank}) \times 100)/Abs_{control}]}$$

In vivo animal models

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 Jandishapur 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 μL 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.

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.

Dimethyl-Benzene-Induced Inflammation Model

Anasthesia 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.

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. (Ujhelyi, J et al 2014). SNEDDS-PL treatment was performed 30 min before starting time of ear edema induction. Data were expressed in micrometers.

7.2.5. Statistical analysis of SMEDDS/SNEDDS compositions

Statistical analysis in BIP-SMEDDS

Data were analyzed using SigmaStat (version 3.1; SPSS, IBM Inc, New York, NY, USA) and presented as means ± 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.

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 ±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 ($n = 5$).

7.2.6. Contributions

- Characterization of Plantago lanceolata and the 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.

8. RESULTS 1.

8.1. SNEDDS containing different antitumor agents

8.1.1.Preparation and investigations of SNEDDS-antitumor agents

In this study Kolliphor RH 40 and Labrasol were used as surface active agents, Capryol 90 or Lauroglycol FCC and Transcutol HP as co-surfactants in each composition at different ratios. The surfactants and co-surfactants were selected on the basis of their emulsification efficiency and ability to solubilise cytostatic agents.

As our SNEDDS compositions were intented to be diluted in aqueous phase to obtain microemulsion, we had to use a hydrophilic surfactant. We used Kolliphor RH 40 as a surfactant and Transcutol HP as a co-surfactant in different ratios, which gave the BIP-SNEDDS optimal emulsification and flowability properties. Six SNEDDS formulations (listed in table 5) were evaluated for self micro-emulsifying performance. The self nano-emulsifying performance of SNEDDS compostions were evaluated on the basis of their pseudoternary phase diagrams and the time taken to produce fine microemulsions. The pseudoternary phase diagrams indicated that microemulsion was generated in each composition. Figure 16 shows the largest microemulsion formation zones observed. Figure 17 presents the distribution of the mean globule size of SNEDDS after dilution with different dissolution media. Following a gentle agitation in distillated water at room temperature, spontaneous microemulsion formation occurred in each of the formulated SNEDDS compositions (see table 5) in not more than 5 minutes after dilution. The percentage of transmittance and the refractive index of the resulting product were 976.8% ± 1.31% and 1.337% ± 0.13%, respectively, which indicates that these formulations were transparent. Furthermore, the created formulations were translucent in appearance, preserved their stability for one month at room temperature and did not show any signs of phase separation. On the basis of the average diameters, composition 3 (1:2:1:6:2 ratio of Isopropyl myristate, Kolliphor RH40, Capryol 90, Transcutol HP and Labrasol) showed that using Transcutol HP resulted in a wider microemulsion region and the smallest droplet size (78.4 nm).

The efficiency of drug encapsulation was determined by the method developed by Q. Li and colleagues (Li, Q. et al, 2015). The encapsulation efficiency of our SMEDDS was evaluated using a model drug in a previous investigation. On the basis of the satisfactory

results of this test, we decided to use these SMEDDS compositions for the formulation of these antitumor drugs.

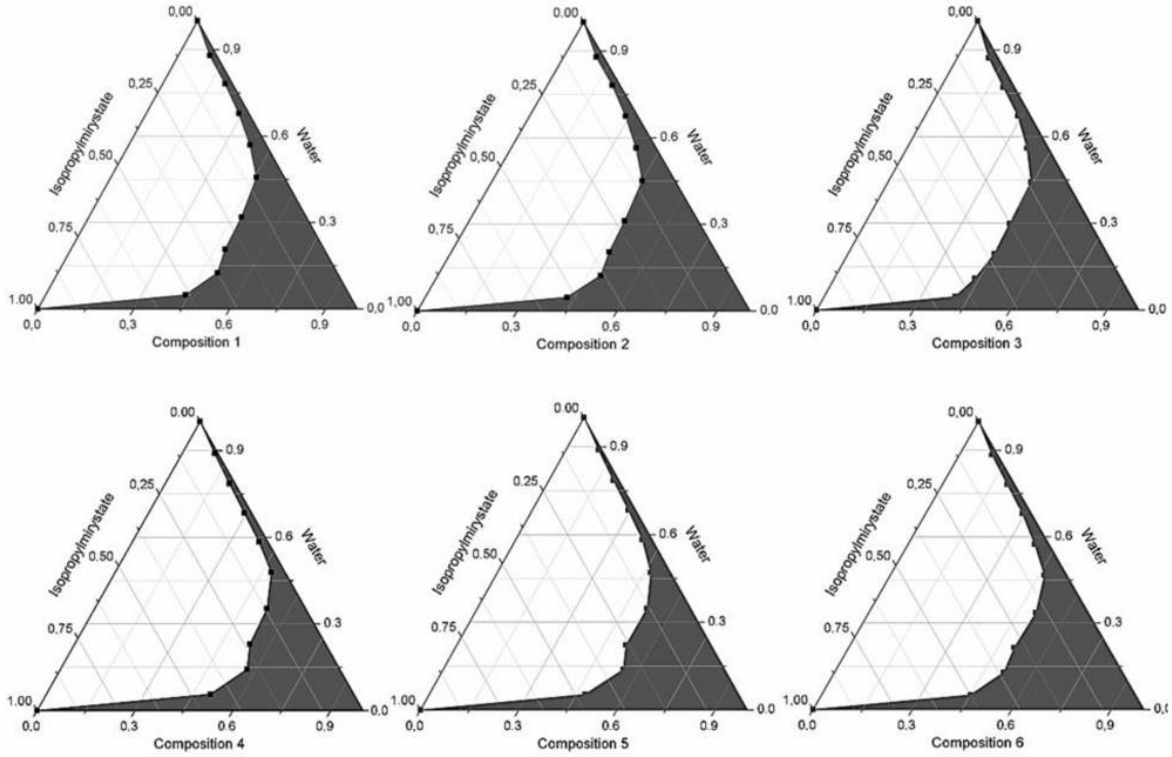


Figure 16. Pseudoternary phase diagrams ofdifferent selected compositions 1-6 dispersed in water at 25°C (Shaded areas represented oil in water microemulsions region).

8.1.2. Droplet Size and Zeta Potential Determination of SNEDDS

We confirmed that increasing the concentration of the surface active agent results in clearer formulations. Moreover, since the o/w interface is stabilized by the surfactant, it is present in a higher concentration at the interface while the oily content is decreased; and as a result of the decreased droplet size, a more transparent nanoemulsion is formed. We determined the droplet size of SNEDDS in water by dynamic light scattering measurements. The evaluated average droplet sizes are as follows: composition 1: 137.81 ± 1.25 nm, composition 2: 68.44 ± 1.05 nm, composition 3:73.28 ± 0.95 nm, composition 4: 129.31 ± 1.95 nm, composition 5: 149.35 ± 1.00 nm, composition 6: 76.90 ± 1.00 nm. We found that all compositions were monodisperse systems (Figure 17) with zeta potentials ranging from −5.01 ± 0.49 mV to −6.55 ± 0.099 mV. The measured values of zeta potential are as follows: composition 1: −5.91 ± 0.87 mV, composition 2: −5.82 ± 0.65 mV, composition 3: −6.55 ± 0.99 mV, composition 4: −5.56 ± 0.32 mV, composition 5: −5.14 ± 0.71 mV,

composition 6: −5.01 ± 0.49 mV. Results are given as means ± SD, *n* = 5.

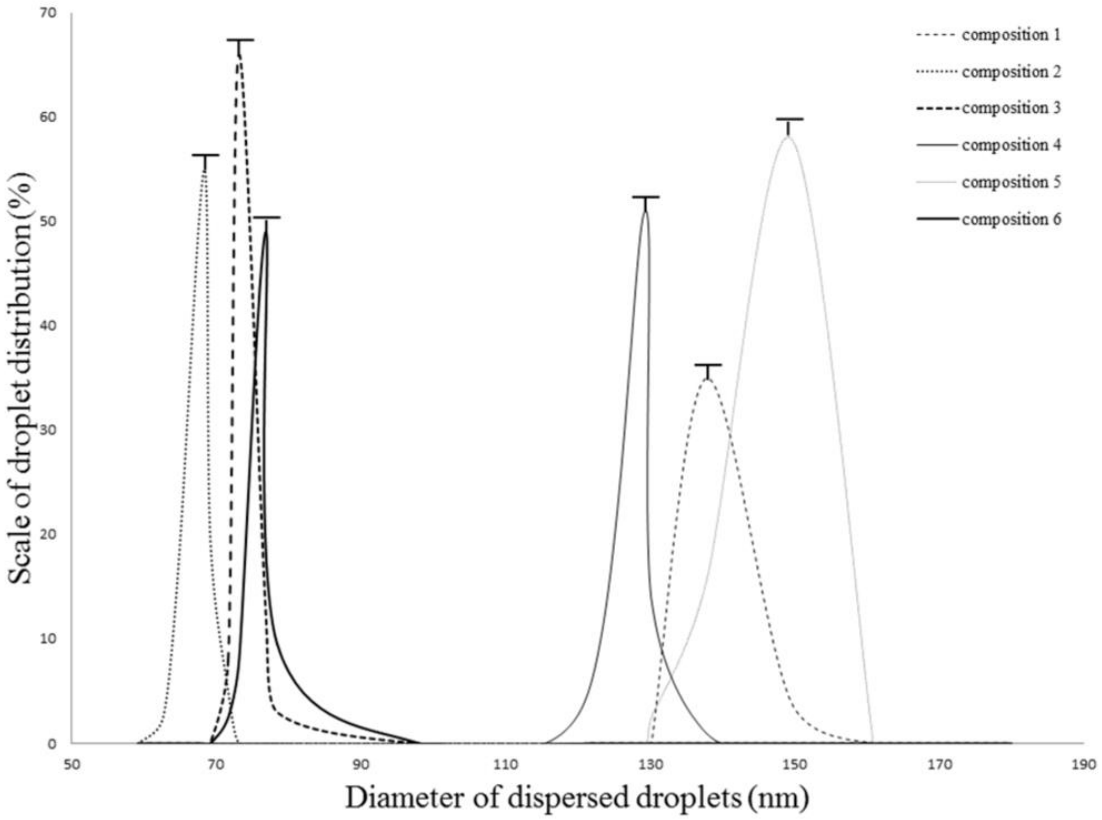


Figure 17.Evaluated droplet size of SNEDDS in water via DLS measurement. Values are expressed as means ± SD, *n*= 5.

Table 7. Evaluated average droplet size and zeta potentials of SNEDDS-BIP combinations

	Average droplet size	**Zeta potential values**
Composition 1.	137.81 ± 1.25 nm	−5.91 ± 0.87 mV
Composition 2.	68.44 ± 1.05 nm	−5.82 ± 0.65 mV
Composition 3.	73.28 ± 0.95 nm	−6.55 ± 0.99 mV
Composition 4.	129.31 ± 1.95 nm	−5.56 ± 0.32 mV
Composition 5.	149.35 ± 1.00 nm	−5.14 ± 0.71 mV
Composition 6.	76.90 ± 1.00 nm	−5.01 ± 0.49 mV

8.1.3. Cell Viability Test of SNEDDS Ingredients

MTT cell viability tests were performed for the evaluation of cytotoxicity in order to exclude that blank SNEDDS components have toxic effects on HeLa cells. Labrasol was

found to be the most toxic agent to HeLa cells (IC_{50}: 0.23 ± 0.025 v/v %), the toxicity of Transcutol HP was lower (IC_{50}: 3.42 ± 0.035 v/v %) and cell viability was reduced in the lowest extent by Kolliphor RH 40 (IC_{50}: 5.12 ± 0.085 v/v %). Nevertheless, the citotoxic effects of surfactants are concentration-dependent, and we obtained significantly higher IC_{50} values from the tests than the concentration range of these componds that was used in SNEDDS formulations (Figure 18).

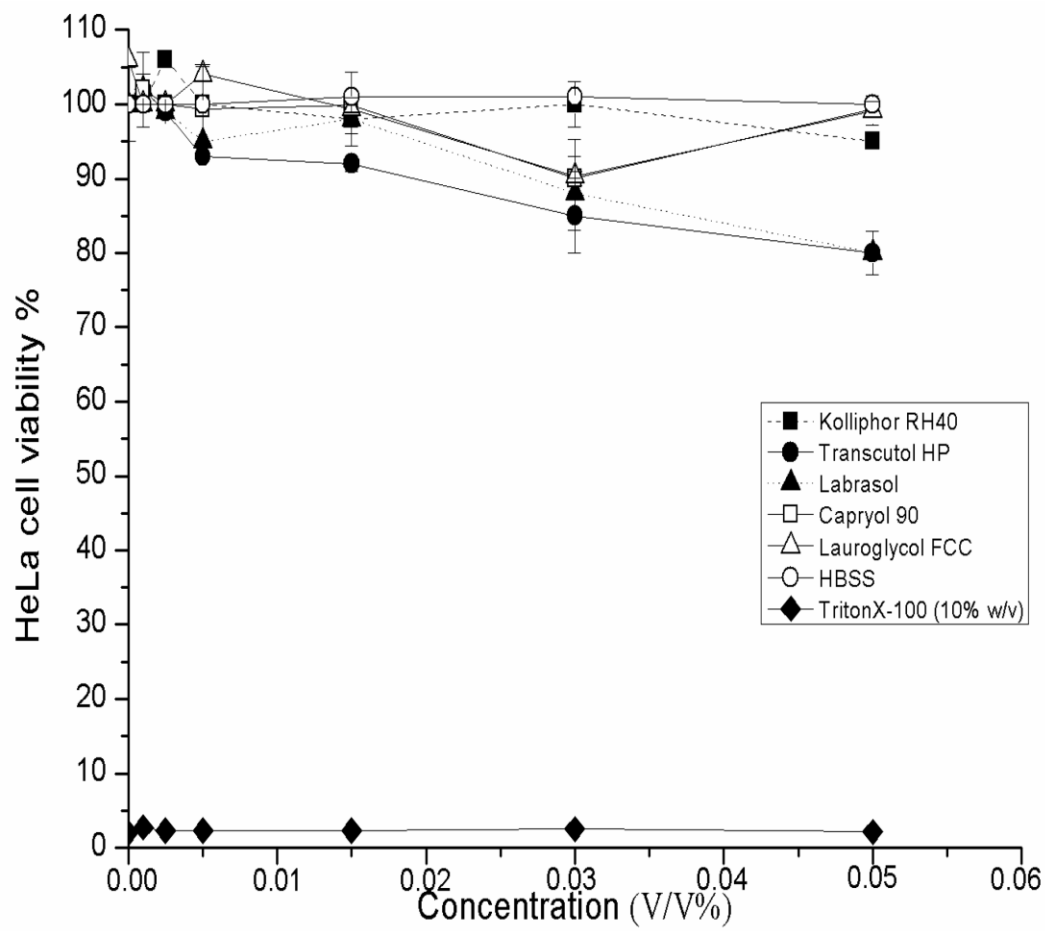


Figure 18. Cytotoxic effects of applied SMEDDS components (surfactant and co-surfactants) on HeLa cells determined by MTT-test. Cell viability was expressed as the percentage of untreated control in the function of surfactant concentration. Positive control: Triton X 100 (10% w/v). Values are expressed as means ± SD, *n* = 5.

8.1.4. Evaluation of Inhibitory Effect of SNEDDS Containing Antitumor Agent

Figure 19 presents the inhibitory effects of cisplatin, bleomycin sulfate and ifosfamide on human cervical cancer HeLa cells when applied alone and in 6 different SNEDDS formulations. On the basis of on our results, the anticancer drugs inhibited cell proliferation in a concentration-dependent manner. Since self-nanoemulsifying drug

delivery systems are lipid-based carrier systems with the ability to increase the solubility and stability of a drug in dissolved state, they are capable to modify the permeability of various drugs by their high surfactant content which enhances penetration. By improving both paracellular and transcellular drug uptake, which results in increased bioavailability, SNEDDS formulations can enhance the antiproliferative effects of cisplatin, bleomycin sulfate and I-fosfamide. The highest level of inhibition was achieved by composition 3 due to its high Transcutol HP content. Bleomycin, cisplatin and ifosfamide were mixed in their respective IC50 concentrations, and this mixture was incorporated into SNEDDS. The inhibitory effects of BIP combinations were greater than those of the anticancer drugs when applied alone.

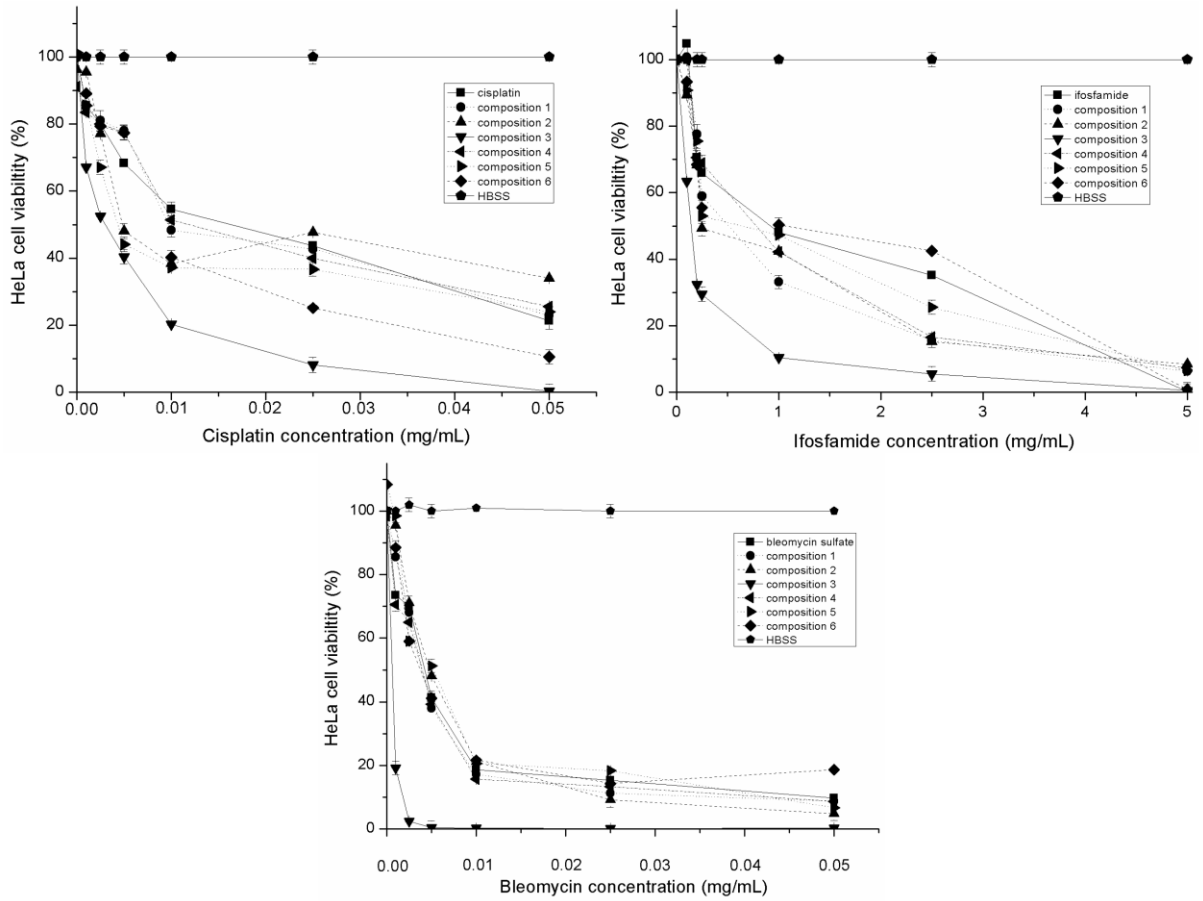


Figure 19. Inhibitory effect of different SNEDDS compositions containing antitumor agent (Bleomycin sulphate, Ifosfamide and Cisplatin) evaluated by MTT cell viability assay. Cell viability expressed as the percentage of untreated control (HBSS). Values are expressed as means ±SD, n=5.

8.1.5. Evaluation of Inhibitory Effect of SNEDDS Containing Antitumor Agent in the Presence of Inflammatory Mediators

Figure 20 presents the inhibitory effects of cisplatin, bleomycin sulfate and ifosfamide on human cervical cancer HeLa cells when applied alone and in SNEDDS formulations in the presence of IL-1-β and TNF-α. The IC_{50} values calculated for these agents. Anticancer drugs in SNEDDS were found to inhibit proliferation to a lesser extent when accompanied by inflammatory mediators. The IC_{50} values of different SNEDDS compositions showed significant differences. The highest level of inhibition was achieved by composition 3 but the IC_{50} value was lower in the presence of inflammatory mediators. Bleomycin, cisplatin and ifosfamide were mixed in their respective IC50 concentrations, and this mixture was incorporated into SNEDDS. The data obtained showed lower IC_{50} values for the combination of anticancer drugs but the effectivity decreased in case of inflammatory mediators being present; it has been confirmed by many studies that inflammation can hinder the efficacy of SNEDDS carriers.

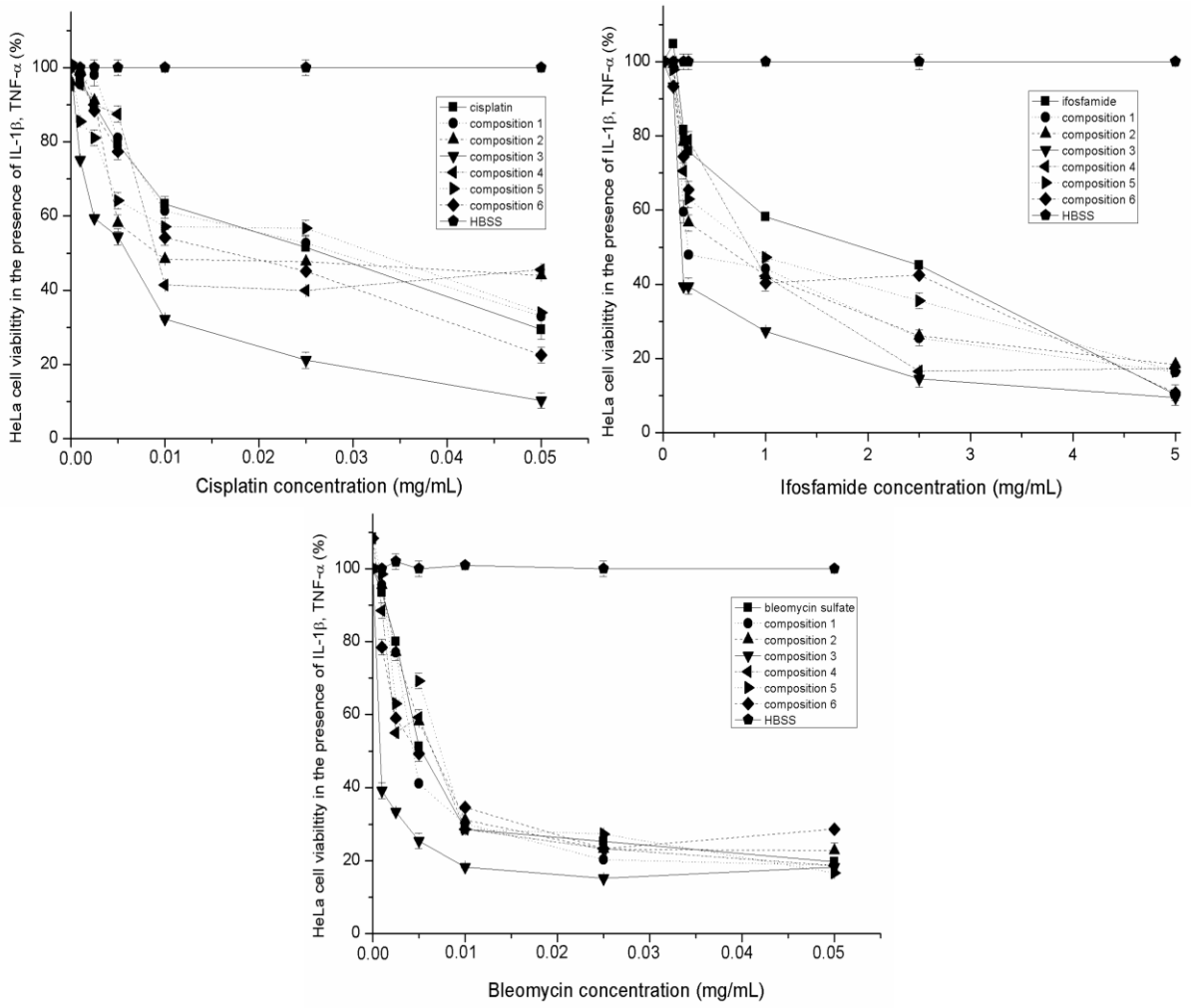


Figure 20. Inhibitory effect of different SNEDDS compositions containing antitumor agent (bleomycin sulfate, ifosfamide and cisplatin) in the presence of inflammatory mediators evaluated by MTT test. Cell viability was expressed as the percentage of untreated control (HBSS). Values are expressed as means ± SD, *n* = 5.

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. It can be seen in the Table 8.

Table 8.: Evaluated IC_{50} values of antitumor agents alone and in combination incorporating in different SMEDDS determined by MTT cell viability assay. Values are expressed as means ± SD, n = 5.

	IC50 (µg/ml)	±SD	IC50 (µg/ml) in presence of TNF-α , $IL1\beta$	±SD
Cisplatin	16.68	0.10	25.96	0.56
Composition 1	9.63	0.11	27.88	0.21
Composition 2	4.81	0.21	8.69	1.00
Composition 3	2.82	0.15	5.84	0.45
Composition 4	11.03	0.96	9.00	0.01
Composition 5	4.26	0.85	32.70	0.21
Composition 6	5.35	0.32	14.29	041
Bleomycin sulfate	4,21	0,11	5,69	0,11
Composition 1	3.84	0.15	4.37	0.19
Composition 2	3.99	0.50	6.70	0.32
Composition 3	0.61	0.012	0.82	0.01
Composition 4	4.71	0.12	6.81	0.01
Composition 5	5.12	0.33	7.56	0.02
Composition 6	4.93	0.22	6.33	0.01
Ifosfamide	993.50	25.00	1936.02	5.40
Composition 1	521.70	20.40	641.10	4.21
Composition 2	247.30	35.60	479.00	4.52
Composition 3	148.50	1.00	191.10	3.66
Composition 4	607.30	10.90	823.20	0.99
Composition 5	802.41	30.55	873.14	0.99
Composition 6	1044.70	1.11	1098.70	1.90
BIP	3.36	0.10	4.93	0.18
Composition 1	2.76	0.23	3.56	0.11
Composition 2	2.01	0.21	3.67	0.34
Composition 3	0.33	0.05	0.95	0.09
Composition 4	3.31	0.08	3.95	0.17
Composition 5	2.99	0.23	4.51	0.35
Composition 6	2.74	0.32	3.45	0.37

8. RESULTS 2.

8.2. Preparation and investigation of different SNEDDS compositions containing Plantago lanceolata extract

8.2.1. Formulation and Evaluation of Self-Nano-Emulsifying Drug Delivery Systems

As it was discussed before, self-nanoemulsifying systems form fine o/w emulsions in aqueous media upon gentle agitation; therefore, selecting the appropriate oil, surfactant and co-surfactant and applying them in the right mixing ratio is of great importance when formulating SNEDDS. In this study, pseudoternary phase diagrams were obtained by utilizing a conventional water titration technique. In our compositions, we used isopropyl-myristate as oily phase, Labrasol or Kolliphor RH 40 as surface active agents and Transcutol HP as co-surfactant. Figure 21 shows the largest microemulsion formation zones observed. The globule droplet size of nanoemulsion formulations were measured by dynamic light scattering (DLS) that employs zetasizer for droplet size analyzing; results are presented in Figure 22 a,b. Evaluated average droplet sizes: composition 1: 233.62 ± 2.34 nm, composition 2: 141.51 ± 1.25 nm, composition 3: 166.44 ± 1.05 nm, composition 5: 374.89 ± 0.23 nm, composition 6: 330.28 ± 0.95 nm, composition 7: 251.81 ± 1.95 nm. Evaluated average droplet sizes: PL-composition 1: 245.32 ± 0.64 nm, PL-composition 2: 121.75 ± 1.00 nm, PL-composition 3: 174.50 ± 1.00 nm, PL-composition 5: 313.02 ± 3.12 nm, PL-composition 6: 459.50 ± 3.85 nm, PL-composition 7: 383.20 ± 2.55 nm. Surfactant/co-surfactant ratio appears to affect droplet size as increasing this ratio results in decreased mean droplet diameter. Following a gentle agitation in distillated water at room temperature, spontaneous microemulsion formation occurred in the developed SNEDDS-PL formulation (see Table 6, Materials part). Each of the compositions contained 10 mg/mL Plantago lanceolata extract. The examined SNEDDS formulations preserved their stability, appearance and microemulsifying property for one month at room temperature while phase separation did not occur. The investigation of SNEDDS also included the determination of the percentage of transmittance and the refractive index. The percentage of transmittance and the refractive index of the resulting formulation were 976.8 ±1.31% and 1.337 ± 0.13% respectively, which indicates that these formulations were transparent. On the basis of these results, eight compositions were selected for further experiments.

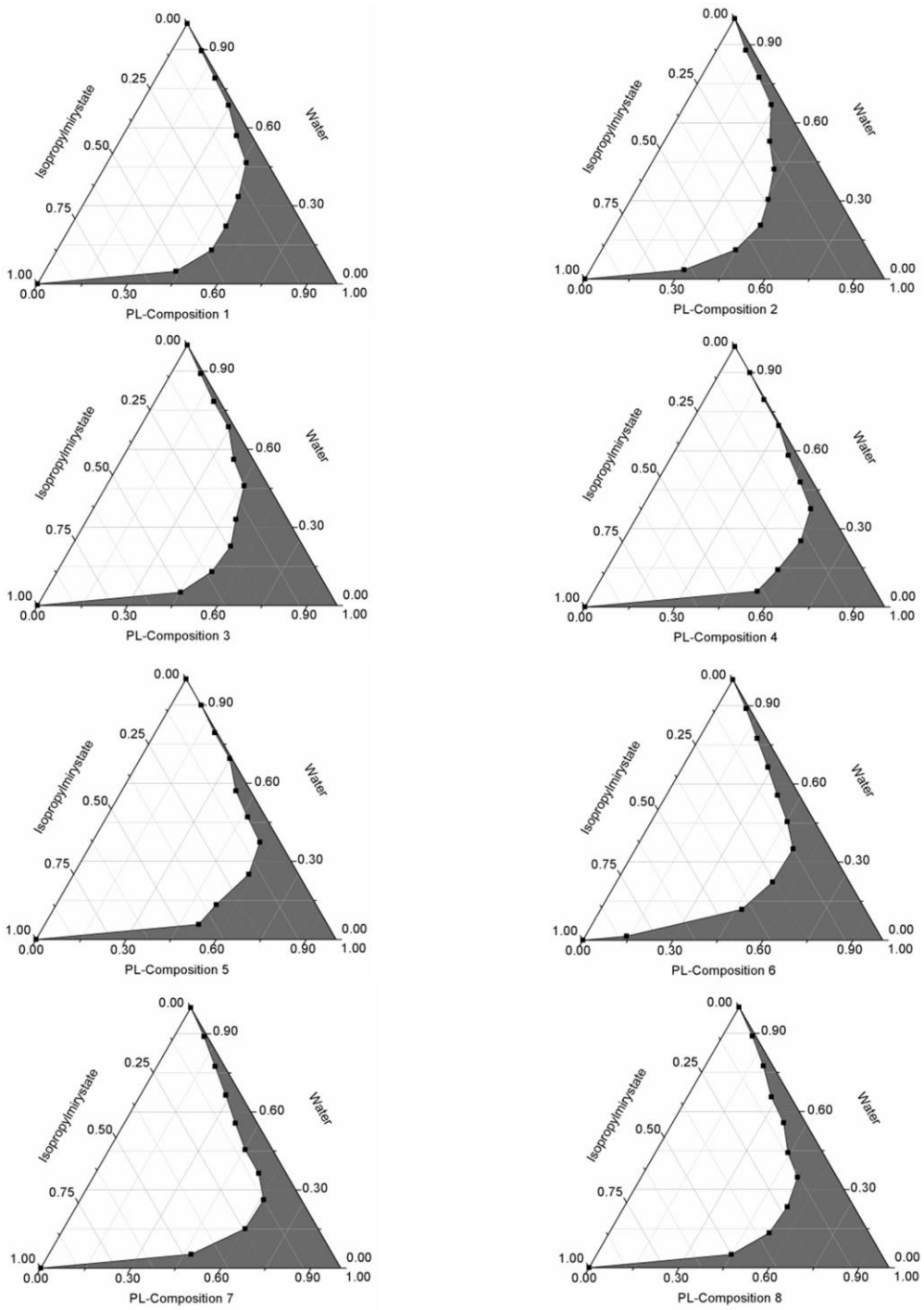


Figure 21. Pseudoternary phase diagrams of PL-Compositions 1–8. (Shaded areas represented nanoemulsions).

Table 9.: Evaluated average droplet size without/with Plantago lanceolate extract

	Average droplet size without Plantago lanceolate extract	Average droplet size with Plantago lanceolate extract
Composition 1.	233.62 ± 2.34 nm	245.32 ± 0.64 nm
Composition 2.	141.51 ± 1.25 nm	121.75 ± 1.00 nm
Composition 3.	166.44 ± 1.05 nm	174.50 ± 1.00 nm
Composition 5.	374.89 ± 0.23 nm	313.02 ± 3.12 nm
Composition 6.	330.28 ± 0.95 nm	459.50 ± 3.85 nm
Composition 7.	251.81 ± 1.95 nm	383.20 ± 2.55 nm

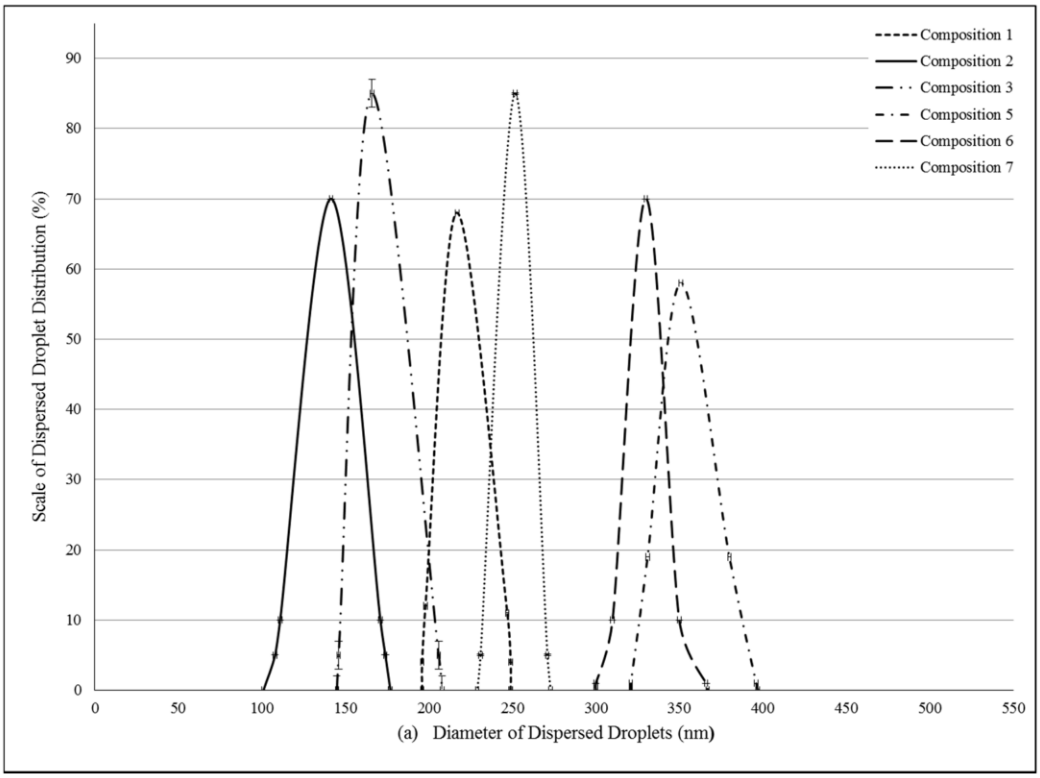
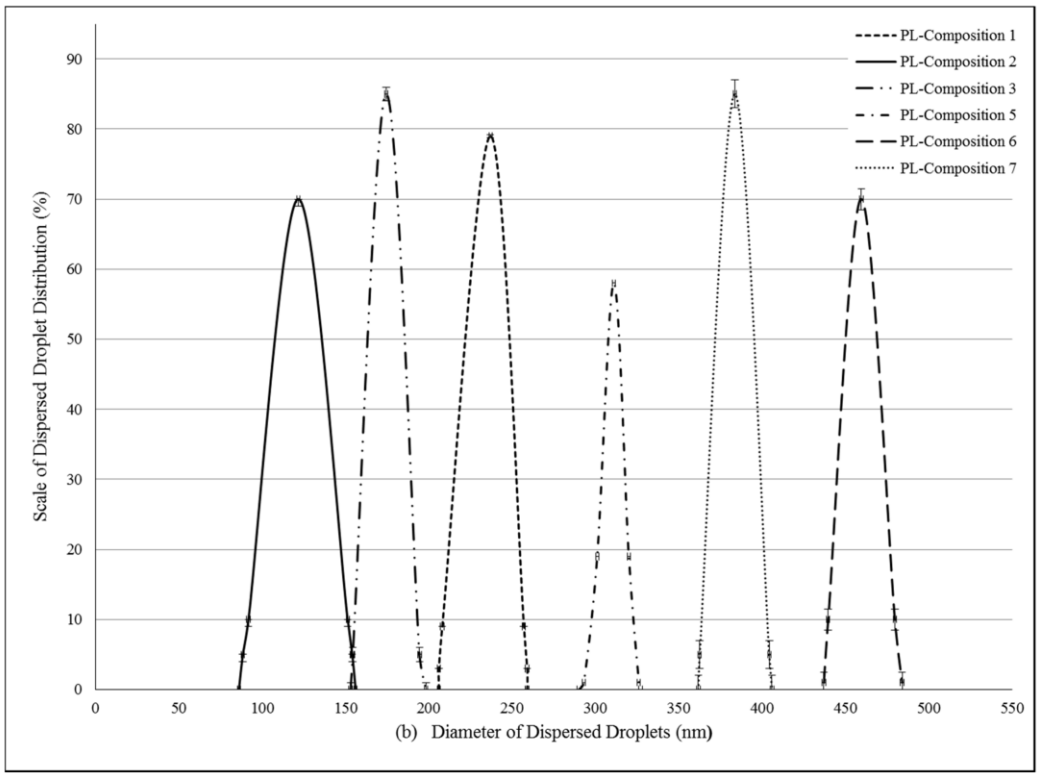


Figure 22. (a) Evaluated droplet size of self-nano-emulsifying drug delivery systems (SNEDDS) in water via dynamic light scattering (DLS) measurement. Values are expressed as means ± SD, *n* = 5;



(b) Evaluated droplet size of self-nano-emulsifying drug delivery systems (SNEDDS) in water via dynamic light scattering (DLS) measurement. Values are expressed as means ± SD, *n* = 5.

8.2.2. Bioactive Compounds in Plantago lancolata Leaves

The concentrations of bioactive compounds present in the leaves of Plantago lanceolata are given in Table 10. 10 mg/mL dry MeOH extract is equivalent to 39.68 mg DW mL/mL plant drug. Compared to typical drug molecules, acteoside is highly hydrophilic (predicted logP = 0.82, logD = 0.81 at pH 7.50). Iridoid glycosides have even stronger polarity (predicted logP = 3.18 and = -3.43 for aucubin and catalpol, respectively).

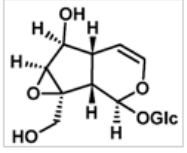
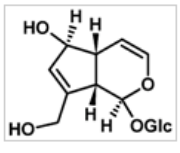
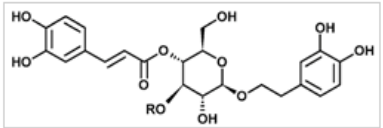
	Catalpol (CA)	Aucubin (AU)	Acteoside/Verbescoside (ACTE)
Chemical structures of bioactive components			
Content in MeOH extract	1.21 ± 0.02%	2.34 ± 0.01%	5.99 ± 0.012%

Table 10. Chemical description of Catalpol, Aucubin, and Acteoside in plantain (*Plantago lanceolata*).

8.2.3. Stability Studies of Self-Nano-Emulsifying Drug Delivery Systems

To evaluate the thermostability of our SNEDDS formulations, we checked the level of the Plantago lanceolata extract in SNEDDS and it was still above 90% after a period of 10 days under controlled conditions of temperature (40°C) and relative humidity (92.5%). When the temperature was raised to 60°C, the P. lanceola extract content dropped to 91.6%. These results indicate that SNEDDS formulations preserve physicochemical stability at high temperatures and humidity. The stability of glycosides found in Plantago species depends on pH. While iridoids undergo spontaneous decomposition under very acidic conditions (basis of the Trim-Hill assay), acteoside loses its stability at pH 7 and above. The stability of the main compounds is maintained at the original pH of the mixture (typically about pH 5–6) to an acceptable degree. It means that the herbal components provide a "buffered" environment in the extract; therefore, most of the compounds are preserved during liquid extraction. Methanol inhibits the activity of the enzyme (beta-glucosidase) which decomposes iridoid glycosides and, in addition, the enzyme is inactivated by heat.

8.2.4. MTT Viability Assay on Caco-2 Cell Monolayers

Caco-2 cells were investigated by MTT cell viability assay (Figure 23 a, b). Hank's balanced solution (HBSS) was used for diluting compositions 1–8. 1 mg PL-SNEDDS composition was diluted by 1–1000 mL HBSS. Cytotoxicity was linearly related to the dilution ratio of the different SNEDDS compositions. Samples with higher concentrations decreased cell viability and caused significant cytotoxicity. The higher the dilution ratio of SNEDDS compositions was, the better the viability of the Caco-2 cells became. When applying the compositions (compositions 1, 2 and 5, 6) with lower Transcutol HP content, higher cell viability values were observed than in the cases of compositions with 60-80% transcutol content (compositions 3, 4 and 7, 8). The compositions had weaker citotoxic effects at a dilution of 1 mg/mL – 1 μg/mL. The differences between the group of compositions 1, 2, 5, 6 and the group of compositions 3, 4, 7, 8 were significant (except for composition 3 vs. compositions 5 and 6 at 100 dilution ratio, where the differences were non-significant statistically). The differences between PL-Composition-treated groups and positive or negative controls are also significant. Regression analysis revealed significant differences between the group of compositions 1, 2, 5, 6 and the group of compositions 3, 4, 7, 8 (Table 11).

	Comp. 1	Comp. 2	Comp. 3	Comp. 4	Comp. 5	Comp. 6	Comp. 7	Comp. 8
B0	59.16	55.65	47.21	39.53	57.13	57.72	44.76	36.85
B1	0.1483	0.1551	0.1984	0.2363	0.15	0.1353	0.187	0.2282
B2	-2.793×10^{-4}	-2.73×10^{-4}	-3.577×10^{-4}	-4.222×10^{-4}	-2.848×10^{-4}	-2.477×10^{-4}	-3.303×10^{-4}	-4.08×10^{-4}
B3	1.738×10^{-7}	1.631×10^{-7}	2.092×10^{-7}	2.414×10^{-7}	1.783×10^{-7}	1.541×10^{-7}	1.924×10^{-7}	2.366×10^{-7}
B0 SE	1.42	1.283	1.766	1.771	1.43	1.283	1.752	1.627
B1 SE	1.785×10^{-2}	1.613×10^{-2}	2.22×10^{-2}	2.226×10^{-2}	1.798×10^{-2}	1.613×10^{-2}	2.202×10^{-2}	2.045×10^{-2}
B2 SE	4.45×10^{-5}	4.022×10^{-5}	5.535×10^{-5}	5.551×10^{-5}	4.483×10^{-5}	4.022×10^{-5}	5.49×10^{-5}	5.1×10^{-5}
B3 SE	2.916×10^{-8}	2.635×10^{-8}	3.627×10^{-8}	3.637×10^{-8}	2.937×10^{-8}	2.635×10^{-8}	3.597×10^{-8}	3.341×10^{-8}
R^2	0.9296	0.9501	0.9252	0.9399	0.9301	0.94	0.925	0.9502

Table 11. Regression parameters using cuboid polynomial equation and goodness of fit data for thecell viability evaluation following MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)assay on Caco-2 cells treated with compositions 1–8 (see: Figure 23, Panel B). SE = Standard Error.(Comp. =Composition).

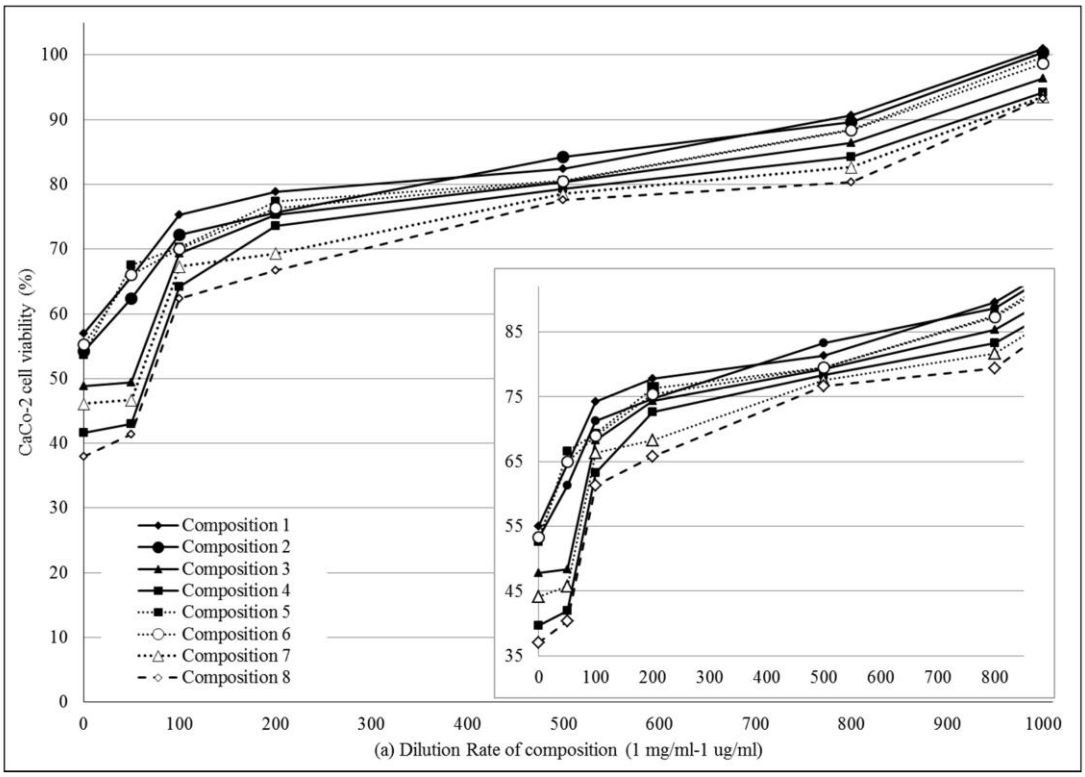
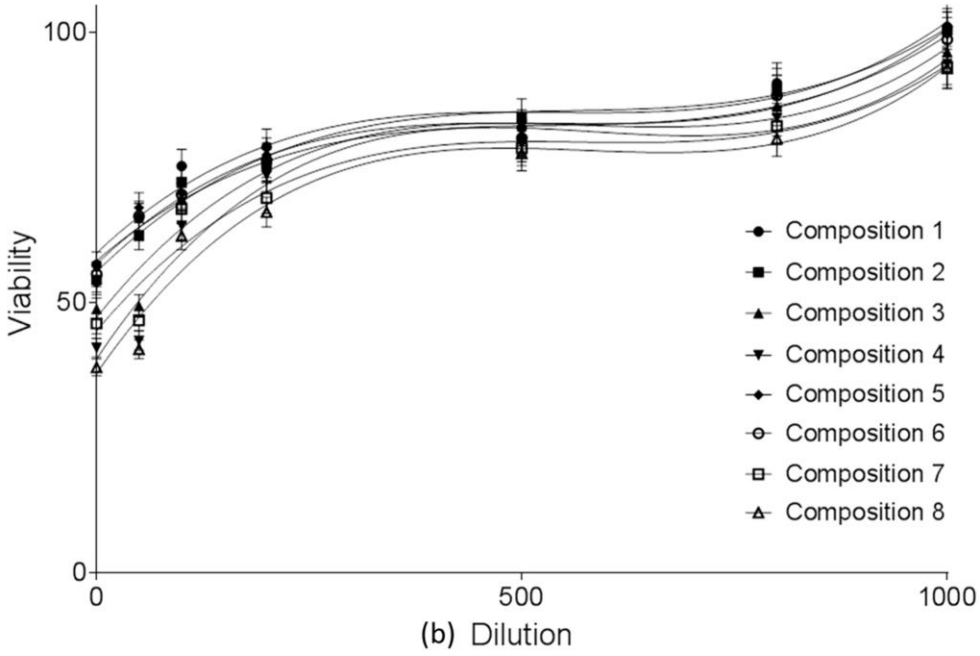
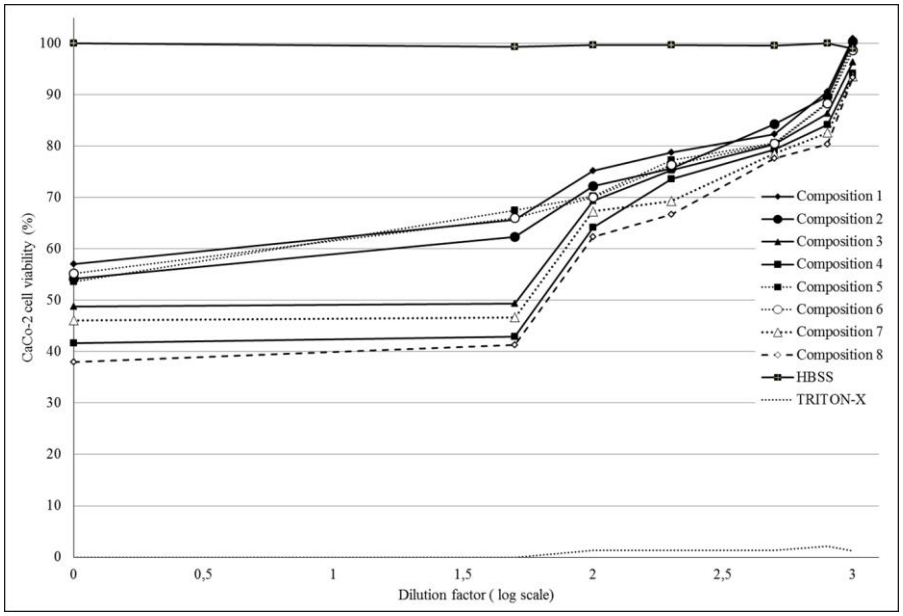


Figure 23. (a) Cell viability evaluation following MTT assay on Caco-2 cells treated with compositions 1–8 in the function of dilution ratio. 1 mg PL-SNEDDS composition was diluted by 1–1000 mL HBSS. Each data point represents the mean ± S.D., *n* = 10. Significant differences between groups for compositions 1, 2, 5, 6 and groups for compositions 3, 4, 7, 8 are marked with an asterisk (with the exception of composition 3 vs. compositions 5 and 6 at 100 dilution ratio, where the differences did not reach level of statistical significance). Positive control was Triton-X-treated group, negative control was Hank's balanced solution (HBSS)-treated group;



(b) Cell viability evaluation following MTT assay on Caco-2 cells treated with compositions 1–8 in the function of dilution ratio. 1 mg PL-SNEDDS composition was diluted by 1–1000 mL HBSS. Each data point represents the mean ± S.D., *n* = 10. Significant differences between groups for compositions 1, 2, 5, 6 and groups for compositions 3, 4, 7,8 are marked with an asterisk (with the exception of composition 3 vs. compositions 5 and 6 at 100 dilution ratio, where the differences did not reach level of statistical significance). Positive control was Triton-X-treated group, negative control was Hank's balanced solution (HBSS)-treated group. Linear regression of cell viability curves presented in the figure 23 (a) (regression parameters and goodness of fit data shown in Table 11).



(c) Cell viability evaluation following MTT assay on Caco-2 cells treated with compositions 1–8 in the function of dilution ratio. 1 mg PL-SNEDDS composition was diluted by 1–1000 mL HBSS. Each data point represents the mean ± S.D., *n* = 10. Logarithmic scale was used for x axis to represent the appropriate IC50 values.

8.2.5. In Vitro Dissolution Study

In vitro release profiles of SNEDDS were obtained by investigating the antioxidant capacity of diffused samples (Figure 24 a, b, Table 12). Plantago lanceolate extract without SNEDDS was used as negative control. There was significant difference between the negative control and each PL-composition-treated group (no mark is shown). There was significant difference in the DPPH inhibition of the dissolved herb extracts from 15 min to 60 min. After 15 minutes, hard gelatin capsules disintegrated, rapid nanoemulsion formation occurred and there was a sharp increase in the antioxidant capacity of PL-compositions. These data indicate that SNEDDS formed nanoemulsion with small droplet size spontaneously after realese from hard gelatin capsule; this resulted in faster API release into the aqueous phase than that of plain Plantago extract. These observations suggest that, due to the dissolved state of plantago lanceolata in SNEDDS, higher rates of drug realese, better absorption and higher bioavailability could be achieved with these compositions. PL-compositions 2 and 6 had significantly higher antioxidant capacity than the other PL-compositions. The results of the DPPH antioxidant assay revealed that the dissolution profiles of the Plantago lancolata extract differed statistically from each SNEDDS. The diffusion of PL-SNEDDS compositions was 4-500% more effective than that of the plain extract.

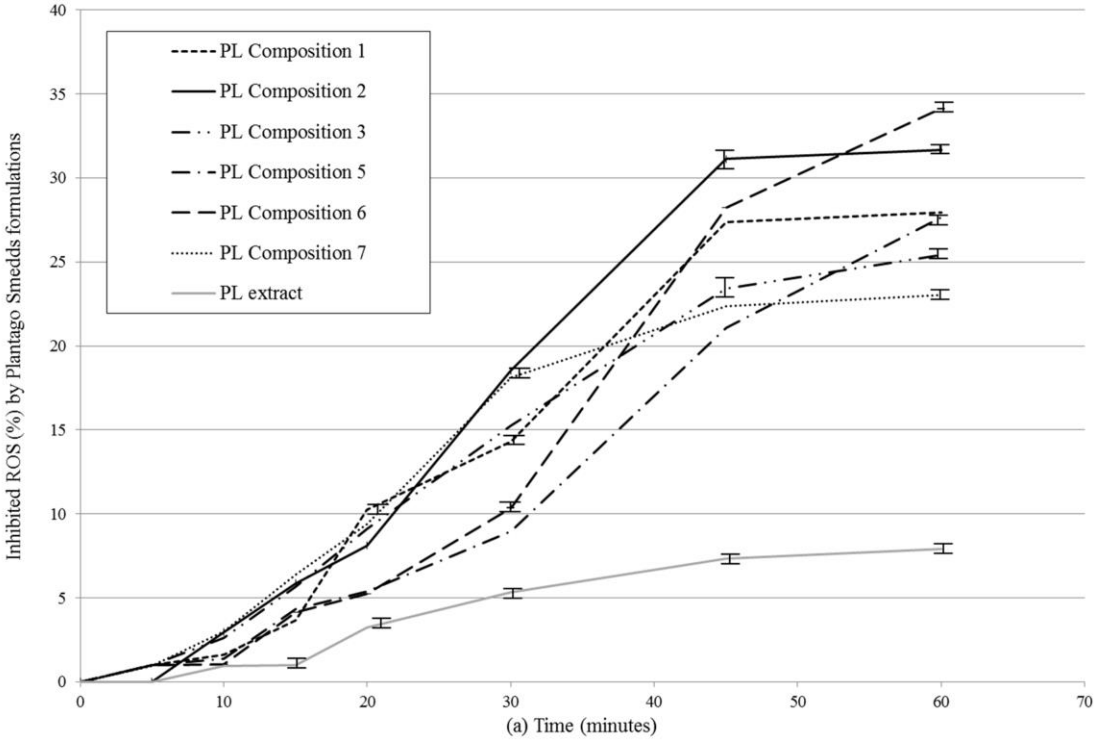
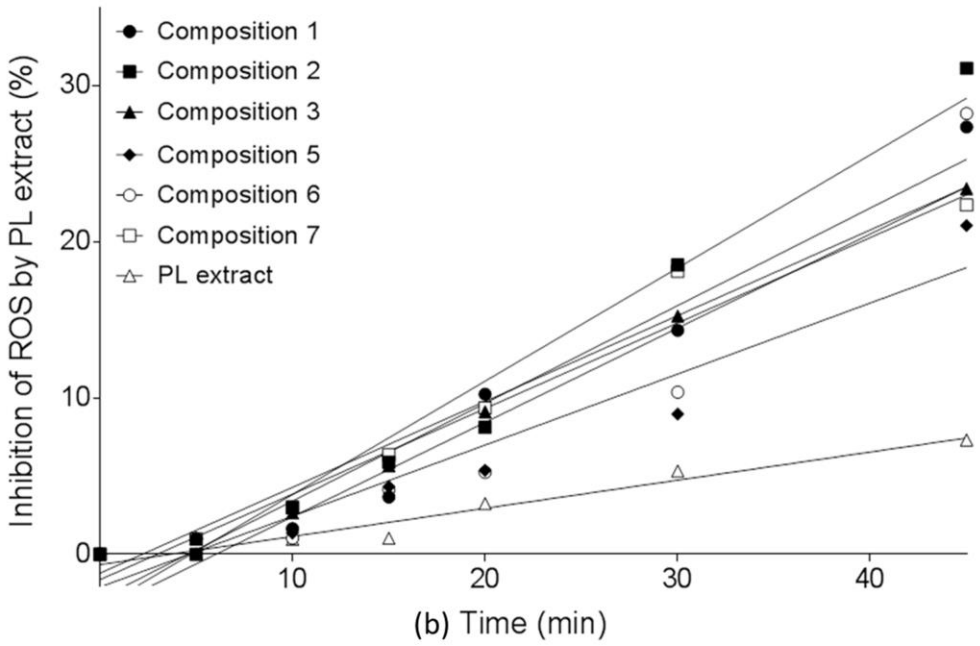


Figure 24. (a) In vitro dissolution study of PL-SNEDDS compositions based on the determination of DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity. Values are expressed as means ± SD, *n* = 5. Negative control was *Plantago lanceolate* extract without SNEDDS. All PL-composition-treated groups differed significantly from the negative control group (no mark is shown);



(b) In vitro dissolution study of PL-SNEDDS compositions based on the determination of DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity. Values are expressed as means ± SD, *n* = 5. Negative control was *Plantago lanceolate* extract without SNEDDS. All PL-composition-treated groups differed significantly from the negative control group (no mark is shown). Linear regression of inhibition curves presented in Figure 24 a. A (regression parameters and goodness of fit data shown in Table 10). As reactive oxidative species (ROS) inhibition data belonging to 60 min obviously deviate from linearity (to the same direction), they were omitted from this analysis.

Table 12. Regression parameters and goodness of fit data for the in vitro dissolution study of PL-SNEDDS compositions based on the determination of DPPH radical scavenging activity (see: Figure 24 b.). SE = Standard Error. (Comp. = Composition)

	Comp. 1	**Comp. 2**	**Comp. 3**	**Comp. 5**	**Comp. 6**	**Comp. 7**	**PL Extract**
Slope	0.625	0.725	0.548	0.454	0.604	0.549	0.18
(±SE)	(±0.061)	(±0.061)	(±0.027)	(±0.056)	(±0.098)	(±0.043)	(±0.017)
Y-intercept	−2.849	−3.436	−1.632	−2.108	−3.635	−1.213	−0.662
(±SE)	(±1.408)	(±1.415)	(±0.627)	(±1.292)	(±2.235)	(±0.988)	(±0.382)
X-intercept	4.556	4.737	2.979	4.638	6.023	2.207	3.677
R^2	0.954	0.9651	0.9877	0.9286	0.8844	0.9702	0.9588

8.2.6. Antioxidant and Anti-inflammatory Activity of Plantago Lanceolata

DPPH Radical Scavenging Activity of SNEDDS-PL Samples

The basis of the DPPH antioxidant assay is the color change of a stable free radical (DPPH) in the presence of antioxidants. We measured the antioxidant capacity of compositions 1–3 and 5–7 with and without Plantago lanceolata extract. The relevant blank compositions served as controls (compositions 1–3 and 5–7). The Plantago lanceolata extract content was 10 mg/ml in each sample (PL-composition). The percentage of antioxidant activity (AA %) of each compound was determined by DPPH free radical assay. We measured DPPH radical scavenging activity as per the protocol described by Brand-Williams and colleagues (Brand-Williams,W et al, 1995). The results revealed significant differences between the PL-composition treated groups and the compositions without PL; also, significant differences were found between the PL-composition treated groups and the PL-E treated sample (positive control); in the latter case, the significant differences are marked with an asterisk. The formation of yellow color solution after the addition of the extract is an indication of the conversion of free radicals (DPPH) to stable DPPH molecules; this reaction is quantified by absorbance measurments. Each composition was found to inhibit DPPH mean oxidation significantly. Among the PL-compositions, PL-composition 6 exerted the strongest antioxidant activity in the DPPH assay. PL-composition 7 was found to exert the slightest activity, but even this low antioxidant capacity was significantly higher than that of the non-formulated Plantago lanceolata extract (PL-E). These results comfirm that Plantago lanceolata is rich in antioxidant compounds (Figure 25).

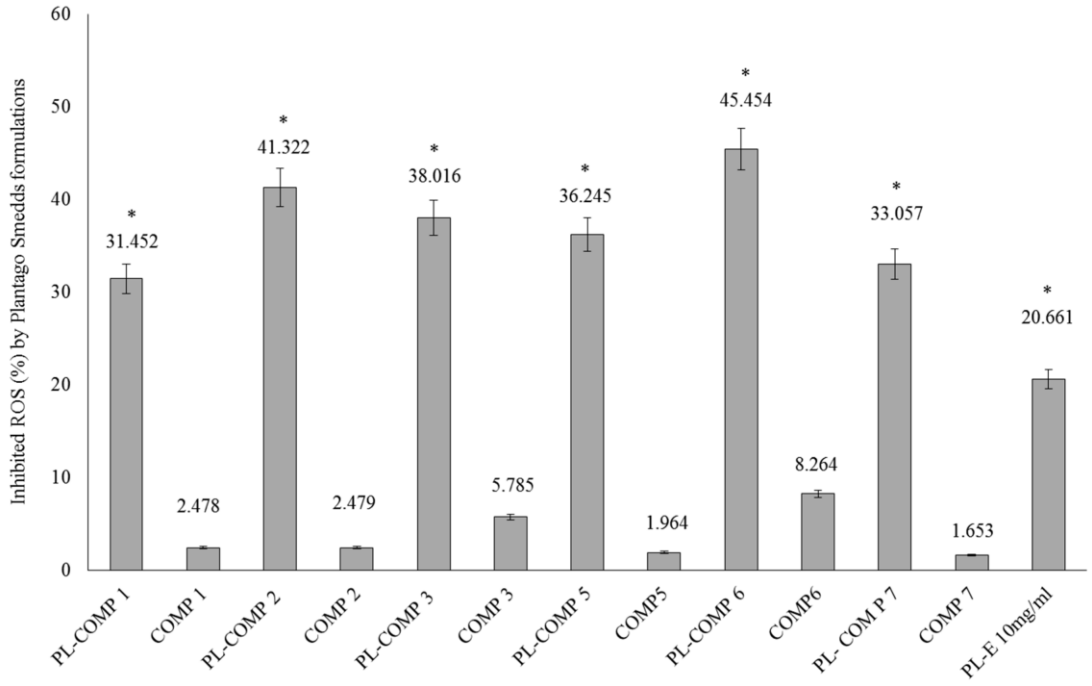


Figure 25. Inhibition of (ROS, %) by different PL-compositions. The positive control was *P. lanceolate* extract in a concentration 10 mg/mL. The negative controls were the compositions without *P. lanceolate* extract. Values are expressed as means ± SD, *n* = 5. Significant differences between PL-composition groups and the positive control group have been marked with an asterisk.

Dimethyl-Benzene-Induced Ear Edema

The rediction of ear thickness was significant after the *per os* administration of each composition with 10 mg/mL Plantago lanceolata extract throughout the complete time period compared to the positive control (dimethyl-benzene alone), as presented in Figure 26. The effect of dimethyl-benzene peaked at 2 h post-challenge time in each case. Throughout the complete examination period, ear edema was reduced by each composition, compared to the positive (untreated) control.

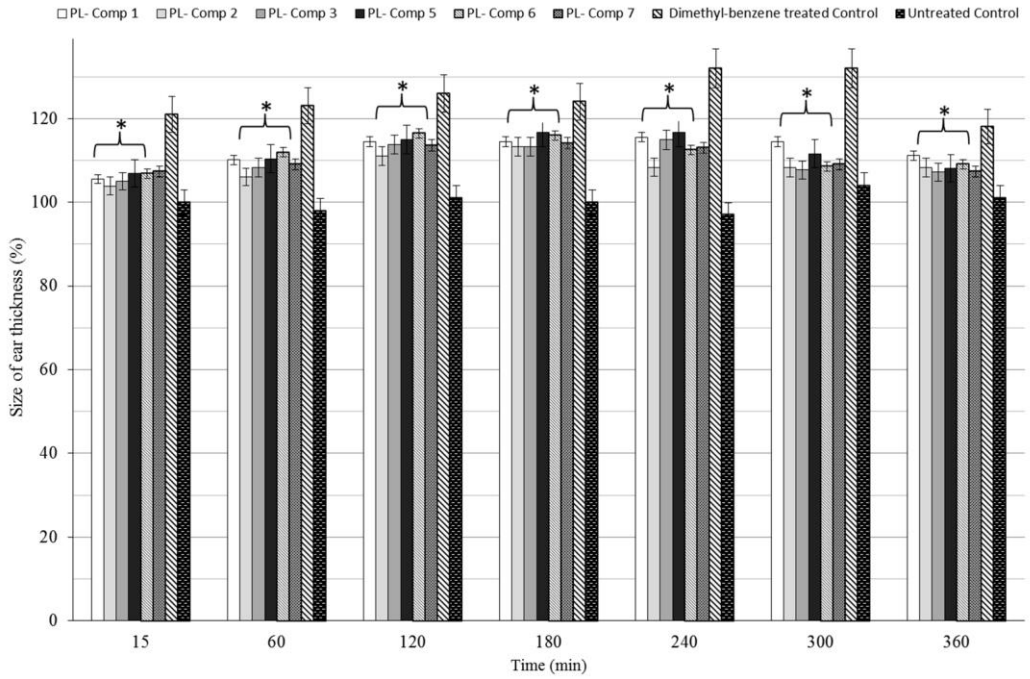


Figure 26. Time revolution of baseline corrected ear thickness (µm) in terms of different SNEDDS compositions containing 10 mg/mL *P. lanceolata*extract in a dimethyl-benzene-induced ear edema model in mice. The administered extract dose was 150 mg/kg/day formulated in SNEDDS and administered by gavage. For the complete time period, compositions showed significant differences to the positive control. As positive control, mice were treated with dimethyl-benzene without PL-SNEDDS pre-treatment, while in the negative control group, the mice received no treatment. Values are expressed as means ± SD, *n* = 6. All groups receiving a treatment with PL-components 1–3, 5–7 differed significantly from their positive control group. Asterisk shown these significant differences.

8.2.7. Effect of PL-SNEDDS on Hepatic Function Markers

The effects of each Plantago-containing SNEDDS composition upon hepatic function markers were tested. The effects of SNEDDS-PL on liver function enzymes AST and ALT two days after per os administration is presented in table 13. The administration of compositions 4 and 8, containing 80% Transcutol HP, led to the death of 5/5 and 4/5 mice, respectively. These compositions were excluded from further experiments. On the other hand, compositions with 33-60% Transcutol content (SNEDDS-PL 1, 2, 3 and 5, 6, 7) did not increase AST levels significantly. AST enzyme levels decreased significantly. There were no significant changes in the ALT enzyme levels. Compared to the normal control group, ALT levels rose moderately. These results indicate that our compositions, with the exception of compositions 4 and 8, could be applied safely in further investigations.

	Control	Comp. 1	Comp. 2	Comp. 3	Comp. 4	Comp. 5	Comp. 6	Comp. 7	Comp. 8
AST (IU/L)	362.28 ± 12.3	183.6 ± 34 *	225.78 ± 13.4 *	285.90 ± 12.3 *	ND	232.56 ± 10.6 *	214.6 ± 12.3 *	298.78 ± 33.2 *	ND
ALT (U/L)	92.34 ± 23.4	113.23 ± 42.1	98.70 ± 29.7	145.62 ± 38.6 *	ND	93.4 ± 21.4	119.21 ± 13.2	138.23 ± 12.6 *	ND

Table 13. Effect of SNEDDS containing P. lanceolata extract on serum AST and ALT enzyme activities.Each value represents the means ± SD for 6 mice and is expressed in IU/L or U/L. Groups treatedwith the compositions 1–8 were compared to the control group, significant differences were markedwith an asterisk. Administration of compositions 4 and 8 resulted in the death of mice within 2 days(ND = No Data), (Comp. = Composition).

9. DISCUSSION 1.

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 (Elfström, K.M, et al 2015). Self-microemulsifying drug delivery systems (SMEDDS) 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 SMEDDS formulations was studied; these SMEDDS formulations contained anticancer drugs—cisplatin, bleomycin sulfate and ifosfamide—alone and in combination. SMEDDS 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 (Chang, T.C. et al, 2000) but application of topical dosage formulations of antitumor agents seems to be more beneficial (Rahangdale, L.et al 2014). Here, we have successfully developed SMEDDS of low dose combinations of cytostatic agents in order to increase the bioavailability and efficiency. Self-microemulsifying 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 SMEDDS 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 SMEDDS. In vitro paracellular and transcelluar uptake of active ingredients could be improved by surfactants and co-surfactants which further increase the oral bioavailability of certain APIs (Sha, X, et al 2005). Many studies confirmed the benefits of SMEDDS in peroral applications but the topical efficacy of SMEDDS formulations is still to be investigated (Chiragkumar, D. et al, 2014).

The efficacy of antitumor agents loaded in SMEDDS 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 SMEDDS, significantly more efficient than alone. In a randomized-controlled study, BIP was approved for advanced and recurrent cervical carcinomas (Tay, S.K et al, 1992). The *in vivo* study revealed that using nanoformulation for the treatment of cervical cancer cells is advantageous considering safety and potency aspects (Saini, J, et al, 2014).

There is a linkage between inflammation and cancer; since inflammation is a critical component of cervical cancer, it can stimulate tumor progression (Deivendran, S. et al, 2014). In this experiment, we also investigated the effects of different SMEDDS 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.

9. DISCUSSION 2.

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 exctract 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 (Isacchi, B et al, 2016). 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 effiectively 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 (Fakhrudin, N et al, 2017). 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 (Li,W. et al 2011), 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 bioavailabiliy of drugs. The characteristics of the applied oil, surfactant and co-surfactant are essential factors in the development self-nano-emulsifying systems (Pouton, C.W, 2000). In our SNEDDS formulation, we used Transcutol HP as co-surfactant which also enhanced penetration effectively (Naguib, S.S et al, 2017); isopropyl-myristate as the oily phase which dissolved the lipophilic components of Plantago lanceolata (Powell, K.C.et al, 2017); 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 (Ujhelyi, Z et al,2013) Amphiphilic molecules are potentially ideal surface active agents and co-

surfactants in microemulsions but to exclude toxicity, it is necessary to perform cytocompatibility screening (Pouton, C.W.2000). 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 (Bigansoli, E.et al, 1999). 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 (Fotakis, G et al, 2006) but the results are more predictive when supported by *in vivo* tests (e.g. liver fuction 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 indicates 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 (parallelogram approach by Xing et al., 2006) (Palamakula, A et al 2004).

10. CONCLUSION

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 compostions containing different antitumor agents on HeLa cells was proven. The inhibitory effect of mixed bleomycib sulphate, cisplatin and ifosfamide incorpareted 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:

- **d.** 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.
- **e.** Plantago lanceolate containing SNEDDS compositions are potentially suitable for improving the stability of bioactive agents of Plantago lanceolate 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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12. KEY WORDS

SMEDDS, SNEDDS, HeLa cell line, Caco-2 cells, Bleomycin, Ifosfamide, Cisplatin, Plantago lanceolata, DLS, cytotoxicity investigation MTT-test, Antioxidant and Anti-inflammatory effects, DPPH test, In vivo ear edema assay, AST/ALT.

13. PUBLICATION LIST RELATED TO THE DISSERTATION

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List of publications related to the dissertation

1. **Kalantari, A.**, Kósa, D., Nemes, D., Ujhelyi, Z., Fehér, P., Vecsernyés, M., Váradi, 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.
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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.
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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.
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15. 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 to improve absorption and bioavailability.

Reducing droplet size results in faster and enhanced drug release and increased bioavailability in the case of certain APIs.

In both studies, the API in the developed SM/NEDDS formulation had higher pharmacodynamic potential than alone. The stability of the developed formulation was confirmed by stability studies. The results of our investigations confirmed that SM/NEDDS formulations are potential alternatives to conventional peroral formulations of BIP and Plantago lanceolata extract and may improve the bioavailability of these drugs. Hydrophobic agents can be delivered orally by SMEDDS which can increase the permeability of these components. In this experiment, different topically applied SMEDDS formulations were selected to develop carrier systems for anticancer drugs. The present research highlighted the phases of development (solubility tests, construction of pseudoternary phase diagrams and different evaluation tests) of a robust and stable formulation. In this research, we focused on SM/NEDDS formulations that contain surfactants/co-surfactants with low toxicity and API in low dose; we developed and evaluated them by different *in vitro* and *in vivo* methods. When formulating unstable herbal drugs, it is difficult to find suitable solvents in which they remain stable despite their high hydrophylicity. New synthetic hydrophilic oils and surface active agents can dissolve and stabilize plant compounds by forming SNEDDS. The antioxidant capacity and total phytochemical content of P. lanceolata leaf extract were examined using DPPH assay. Overall, this medicinal plant has a relatively high antioxidant capacity and phytochemical content. It also exerts strong anti-inflammatory activity which is believed to be one of the most important pharmacological properties. The conclusions of this investigation support the view that P. lanceolata plant could be used as an effective anti-inflammatory herbal remedy and an easily accessible source of antioxidants. Formulating SNEDDS is a promising way of dealing with the difficulties of compounds with poor stability.

As a final conclusion, it can be stated that PL-SNEDDS compositions are potentially suitable for improving the stability of the bioactive agents of Plantago lanceolata extract and are also able to enhance the antioxidant and anti-inflammatory effects of plant compounds. These formulations could be used as appropriate alternatives for therapeutic purposes. However, based on the results of *in vitro* cytocompatibility and *in vivo* animal studies, it is necessary to perform further tests to evaluate the overall toxicity profile of PL-SNEDDS compositions. It can also be stated that topically applied self-microemulsifying drug delivery systems which contain various anticancer drugs may become therapeutic alternatives for cervical cancer. The efficiency of the treatment can be hindered by inflammatory mediators. Our investigations may contribute to the development of optimal anticancer formulations with valuable data.