Thesis for the degree Master of Pharmacy

DEVELOPMENT OF LIPOSOMAL CURCUMIN FOR VAGINAL DRUG DELIVERY

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ABSTRACT

Curcumin (I), demethoxy curcumin (II) and bisdemethoxy curcumin (III) are commonly called curcuminoids, and derived products from the spice, turmeric. It has reported numerous of therapeutic activities including, anti-inflammatory, and anticancer properties. The aim of the current study was to develop a formulation which can overcome the limitation of curcumin being so poorly soluble in aqueous medium. Our approach has been directed toward investigating the potential of using liposomal formulations as carrier system for curcumin destined for treatment of vaginal inflammation. Curcumin containing liposomes were prepared using soya phosphatidylcholine by the modified film method. Moreover, we added cholesterol in various molar ratios to affect the vesicle membrane rigidity. Curcumin entrapped in the liposomes was quantified and the entrapment efficiency was found to be reaching up to 100%. The size and size distribution of liposomes were determined on photon correlation spectroscopy. The results showed an increase in size of liposomes containing curcumin in comparison with empty liposomes. The accelerated stability testing was used to predict the stability of the formulations. The test revealed changes in the characteristics of the liposomes. The free radical scavenging activity (DPPH) assay of curcumin and Curcuma extract, as well as isolated pure curcumin I, revealed that curcuminoids mixtures have stronger activity.

ABBREVIATIONS

Chol	Cholesterol
DPPH	1, 1- diphenyl-2-picryl hydrazyl
EC50	Effective concentration required to reduce DPPH radical by 50%
E.E.%	Entrapment efficiency
HPLC	High performance liquid chromatography
Lipoid S-100	Soya phosphatidylcholine containing 100% PC
LUVs	Large unilamellar vesicles
MLVs	Multilamellar vesicles
PC	Phosphatidylcholine
PC/Chol (2:1)	Mixture of phosphatidylcholine and cholesterol with molar ratio 2:1
PC/Chol (4:1)	Mixture of phosphatidylcholine and cholesterol with molar ratio 4:1
PCS	Photon correlation spectroscopy
Rec.	Recovery
P.I.	Polydispersity index
SD	Standard deviation
SEC	Size exclusion chromatography
SUVs	Small unilamellar vesicles
vol/vol	Volume ratio
w/w	Weight ratio

1. INTRODUCTION

1.1. Vagina as Site for Drug Therapy

The effectiveness of the vagina as a site of drug administration for local effects has been well established (Jain et al., 1997; Pavelic et al., 2001). It is an important route for local treatment of several gynecological conditions, such as infections and in hormonal therapy. This route provides advantages such as reducing or eliminating the incidence and severity of side effects, being a non-invasive route of administration and accessibility. These benefits could contribute to a better compliance, thus achieving improved therapeutic outcome (Knuth et al., 1993; Pavelic et al., 2004a). Furthermore, the vagina possesses properties which include: large surface area of the vaginal wall, permeability, a rich blood supply and importantly, the ability to bypass first-pass liver metabolism (Vermani and Garg 2000; Pavelic et al., 2001). These properties are considered to be advantageous in relation to drug absorption.

Currently, there is a variety of pharmaceutical products available on the market designed for intravaginal therapy (tablets, creams, suppositories, pessaries, foams, solutions, ointments and gels). However, their efficacy is often limited by a poor retention at the site of action due to the self-cleansing action of the vaginal tract (Pavelic et al., 2001). Furthermore, the vagina has unique features in terms of microflora, pH and cyclic changes, and these factors influence the performance of the formulations and must be considered during the development and evaluation of vaginal delivery systems (Valenta, 2005). Therefore, a successful delivery of drugs through the vagina represents a pharmaceutical challenge.

1.1.1. Anatomy and Physiology of Vagina

The human vagina is a tubular, fibromuscular organ that extends from the cervix of the uterus to the vaginal vestibule measured in a length of approximately 9 cm (das Neves and Bahia, 2006). The vaginal blood supply comes from the internal iliac arteries branching into a complex network of arteries and veins surrounding the vaginal wall. Blood leaving the vagina enters the peripheral circulation via the internal iliac veins, thus bypassing the liver (Richardson and Illum, 1992; Knuth et al., 1993).

Histologically vagina consists of three distinct layers: an epithelial layer, a middle muscular layer and an outer fibrous layer. The epithelial layer is classified as a non-cornified, stratified squamous epithelium. This layer is composed of lamina propria and an epithelial cell layer which contain particulate glycogen (Figure 1). The normal thickness of the vaginal epithelium is approximately 200 µm (Richardson and Illum, 1992). It is usually considered to be a mucosal surface, although it has no goblet cells and lacks the direct release of mucins (Robinson and Bologna, 1994; Hussain and Ahsan, 2005; Valenta, 2005). The surface of vagina has a number of folds or also called rugae, which increase the surface area of the vaginal wall (Hussain and Ahsan, 2005).

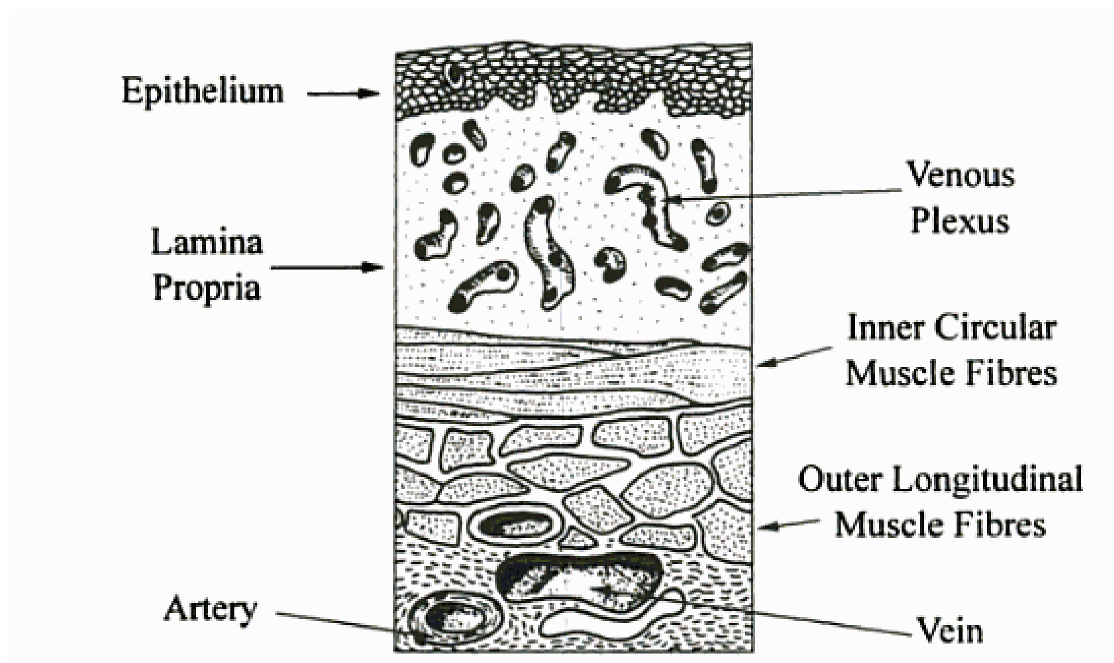


Figure 1: Schematic illustration of the vaginal wall (Washington et al., 2001).

Despite of the absence of any glands, the vagina produces a large amount of fluid (Hussain and Ahsan, 2005). This fluid is a mixture of several components and includes leukocytes, inorganic and organic salts, mucins, proteins, carbohydrates, urea and fatty acids (Vermani and Garg, 2000). Like the thickness of the epithelial layer, the amount and composition of the vaginal fluid change upon the hormonal activity (Robinson and Bologna, 1994; Hussain and Ahsan, 2005).

The lactobacilli bacteria are an important component of the vaginal microflora. This bacteria converts glycogen from exfoliated epithelial cells into lactic acid, and as a result, maintains the pH around 4-5, with lowest being around the cervix. Body fluids such as menstrual blood, cervical and uterine secretions will all act as alkalizing agents and increase the vaginal pH (Richardson and Illum, 1992).

1.1.2. Vaginal Environment

The volume, viscosity and pH of vaginal fluid as well as the thickness and porosity of epithelial layer may have either negative or positive impact on vaginal drug absorption (Hussain and Ahsan, 2005). Therefore it is crucial to understand the conditions that might influence these parameters. It is noteworthy that the histology and physiology of the vagina may vary with age and with the menstrual cycle. Post-menopausal women experience important changes in the vaginal physiology. These changes manifest as decline in estrogen production during the pre-menopause and ongoing menopause. This leads to a permanent decrease in the vaginal glycogen content, and consequently thinning of the vaginal epithelium (Valenta, 2005). Furthermore, elevation of vaginal pH to 6.0–7.5, and a decrease in the quantity of vaginal secretions have been reported (das Neves and Bahia, 2006). It was estimated that the vaginal secretion produced by postmenopausal women is reduced by 50% compared to that produced by women of reproductive age (Washington et al., 2001; Hussain and Ahsan, 2005).

Menstruation is another physiological factor associated with hormonal events. The epithelial layer changes in thickness by approximately 200-300 µm as estrogen levels change throughout the menstrual cycle (Hussain and Ahsan, 2005). Changes in the vaginal pH and viscosity during the menstrual cycle are results of these changes in vaginal histology and physiology. The vaginal pH tends to be lowest at ovulation when estrogen levels reach a peak and both glycogen accumulation and epithelial desquamation at its maximum (Deshpande et al., 1992; Richardson and Illum, 1992).

These physiological cyclic variations will be affected by the use of oral contraceptives. Progestin-containing formulations are associated with the production of viscous mucus

throughout the treatment cycle (Richardson and Illum, 1992). The vaginal pH can be altered by pathological conditions such as infections and inflammations. Diseases such as candidal vaginitis may lower the pH to below 4.5, whereas the inflammatory vaginitis could elevate the pH to over 6.0 (Deshpande et al., 1992; Milani et al., 2000).

1.1.3. Changes in Inflammation

The inflammation is a complex reaction representing the host defenses to microbial infection and irritations. Inflammation may take place in two stages, acute and chronic, and mediates tissue repair and regeneration which may occur due to infectious or non-infectious tissue damage (Kumar et al., 2010).

The acute phase of inflammation has a rapid onset and usually lasts for short duration, hours or a few days; and consists of mainly the responses of blood vessels and leukocytes infiltration.

Chemical irritations may initiate inflammatory reactions and cause membrane damage of the mucosal epithelial cells, which is followed by release of prepackaged cytokines. Mediators initiate and amplify the inflammatory response and cause arteriolar dilation, which results in an increase of the blood flow to the injured area. Altering the permeability is another effect of these mediators. Increased permeability results in plasma proteins leaving the vessels through widened interendothelial cell junctions and cause accumulation of protein-rich extravascular fluid. Moreover, circulating leukocytes adhere to the endothelium via adhesion molecules and infiltrate to the site of injury under the influence of chemotactic agents (Kumar et al., 2010). When acute inflammation is successful in eliminating the offenders, the reaction subsides through activation of endogenous anti-inflammatory response mediated by agents such as interleukin-13 and interleukin-16 (Fichorova et al., 2005). However, if the response fails to clear the invaders, inflammation can progress to the chronic phase.

Chronic inflammation has longer duration. In this phase leukocytes that are activated by the offending agent and by endogenous mediators may release toxic metabolites and proteases extracellularly resulting in damaging tissue. Furthermore, this phase is associated

with the presence of lymphocytes and macrophages, the proliferation of blood vessels, fibrosis, causing destruction and remodeling of the tissue (Kumar et al., 2010).

The inflammatory reaction within the female reproductive tract may be essential for immune responses in clearance of infections and offending agents. However, if this reaction enters the chronic phase and persists, there will be higher probability for complications to arise.

Numbers of studies have linked chronic inflammation with increased risk of viral infections such as human immunodeficiency virus (HIV). Proinflammatory cytokines and the transcription factors controlling the cytokine expression play a major role in HIV-1 pathogenesis (Fichorova et al., 2005). It was reported that continuous stimulation of vaginal epithelial cells by proinflammatory mediators could lead to an increase in the availability of potential host cells for infection and viral replication to occur. Furthermore, it also leads to higher probability of viral transmission during sexual intercourse (Fichorova et al., 2005).

Several inflammatory agents are also linked to cancer promotion and development. An example of these mediators is the tumor necrosis factor (TNF). TNF induces death of diseased cells at the site of inflammation, and stimulates fibroblast growth. However, if this agent is produced chronically, it may act as an endogenous tumour promoter, contributing to the tissue remodelling and tumour growth and spread (Balkwill and Mantovani, 2001).

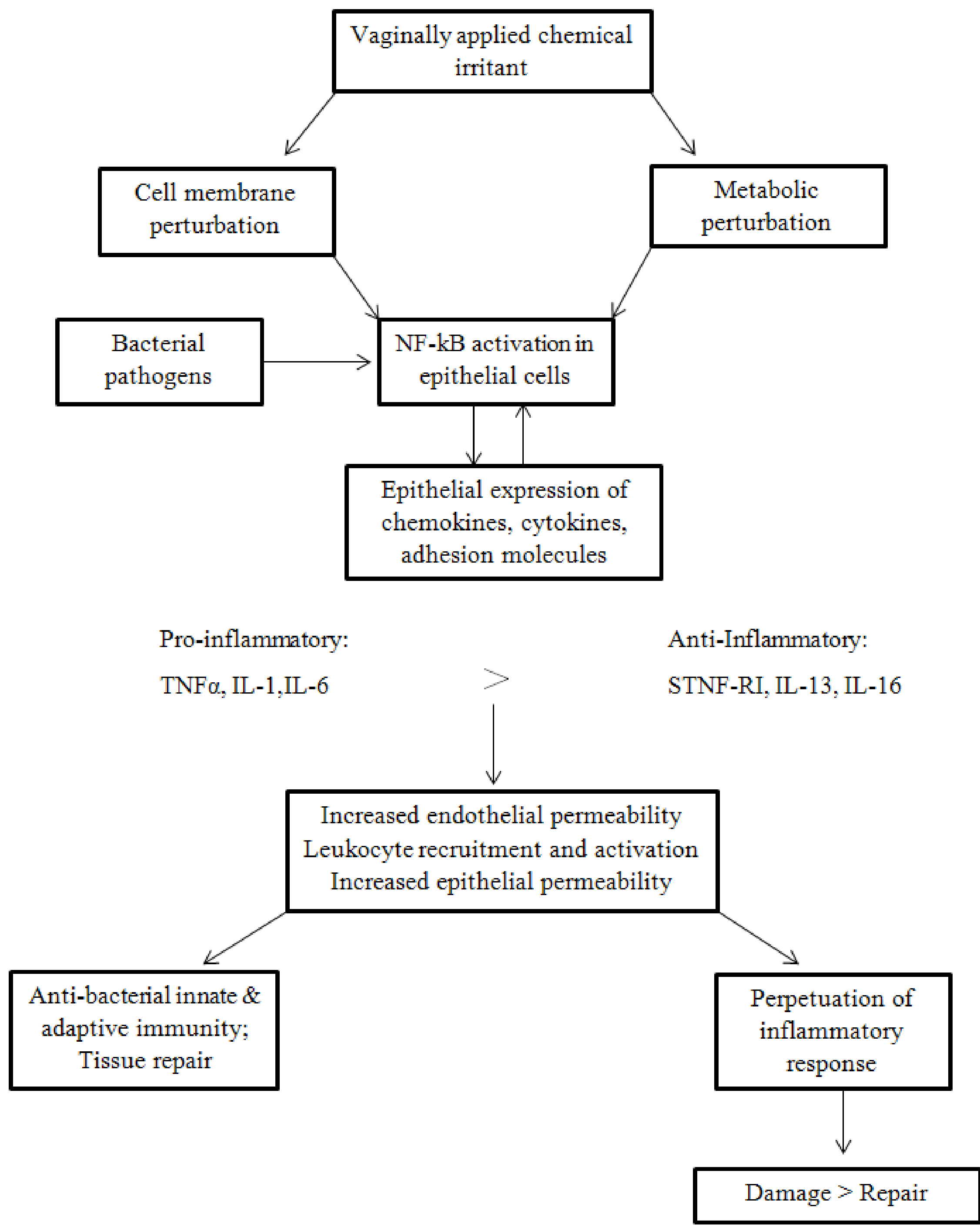


Figure 2: Association between chemical irritation and inflammatory pathways. Abbreviations: TNFα, tumor necrosis factor alpha; STNF-RI, soluble tumor necrosis factor α receptor I; NF-κB, nuclear factor-kappa B; IL, Interleukin. (Fichorova et al., 2005)

1.1.4. Vaginal Dosage Forms

The majority of commercially available vaginal delivery systems are usually targeting topical administration. Pessaries (tablets or suppositories) are among the most widely used systems. The principle of their action is that they provide sustained release of the drug as they gradually dissolve or melt. However, this mechanism has a drawback and can result in low bioavailability if the formulation melted faster than intended, thereby giving shorter residence time in the vagina (Brannon-Peppas, 1993). Vaginal tablets may contain binders, disintegrants and other excipients that are used to prepare conventional oral tablets (Brannon-Peppas, 1993; Hussain and Ahsan, 2005). Formulating very hydrophobic drugs as vaginal tablets may not be an ideal approach. However, it was suggested that by adding penetration enhancing agents such as surfactants can significantly enhance the drug absorption (Hussain and Ahsan, 2005). Moreover, attempts have been carried out to use mucoadhesive polymers in vaginal tablet formulations in order to increase the residence time. Polyacrylic acid (PAA) is among the bioadhesive polymers that have been utilized for vaginal formulations due to its high bioadhesive strength which allows a longer contact time with vaginal surface (Ahuja et al., 1997).

Ideally, vaginal drug delivery system that is designed for local effect should distribute uniformly throughout the site of action. However, the distribution and coverage of formulation within the vaginal cavity varies with the properties of the delivery system. It was reported that disintegrating tablet show low coverage whereas solution, suspension and emulsions display greater distribution profile (Knuth et al., 1993; Washington et al., 2001).

Creams and gels are another type of delivery systems frequently used. Creams are normally emulsions whereas gels are usually hydrophilic polymers that utilize covalent bonds to create cross-linked three-dimensional structures (Hussain and Ahsan, 2005). Some formulations such as antifungal emulsion-based formulations seem to have greater advantage over many suppository formulations (Washington et al., 2001). An example of gel product is the progesterone gel formulation that is based on a loosely cross-linked poly acrylic acid (Noveon AA1®). This formulation was found to remain on vaginal tissue for 3-4 days, thus allowing dosing intervals of twice a week (Knuth et al., 1993). However, a disadvantage that can be associated with the use of creams and gels is that they may not

provide an exact dose, thus compromising the efficacy of the drug therapy (Hussain and Ahsan, 2005).

1.2. Curcumin

The growing public interest in traditional medicine, particularly plants-based medicine, has led to extensive research on the potentials of natural origin substances. Hundreds of studies were conducted to investigate the effects of natural origin compounds on human health and prevention and treatment of chronic diseases (Schmidt et al., 2007). Among studied compounds, polyphenols appear as one of the most promising groups. In plants, polyphenols are important for growth and protection against pathogens. Polyphenols have recently received much attention in disease prevention and treatment due to their proven antioxidant capabilities (Zern and Fernandez, 2005). Polyphenols are derived from many components of the human food including peanuts, dark chocolate, green and black tea and turmeric. Among polyphenols, curcumin is currently one of the most studied substances. It is a hydrophobic, low molecular weight polyphenol widely used in form of the spice, turmeric (Anand et al., 2007; Suresh and Srinivasan, 2007).

1.2.1. Origin

Turmeric has been used in Asia for thousands of years in food, preservation of food, and as traditional medicine (Aggarwal et al., 2007). It is the yellow spice derived from the roots, rhizome, of the plant *Curcuma longa*. The powdered extracts of dried roots, often called turmeric, ukon (in Japanese), or haldi (in Hindi), may contain volatile and nonvolatile oils, proteins, fat, minerals, carbohydrates, moisture and curcuminoids. The curcuminoids which constitute approximately 5% of most turmeric preparations are a mixture of three principal compounds: curcumin (sometimes referred to as curcumin I), demethoxycurcumin (curcumin II), and bisdemethoxycurcumin (curcumin III) (Strimpakos and Sharma, 2008). The majority of commercially available curcumin contains the following composition: curcumin I (77%), curcumin II (17%) and curcumin III (3%) (Goel et al., 2008).

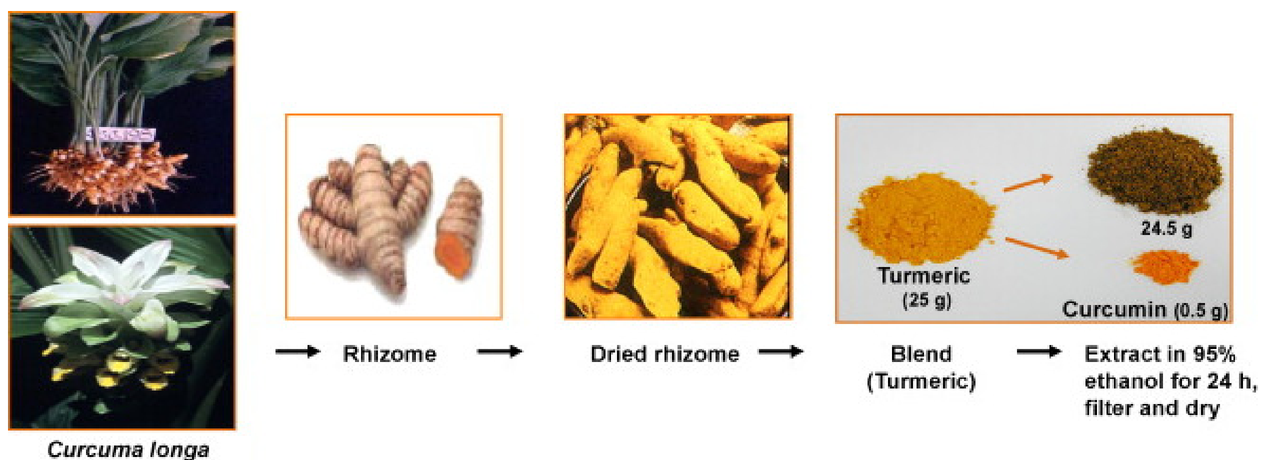


Figure 3: The extraction of curcumin (Goel et al., 2008).

1.2.2. Chemical Properties of Curcumin

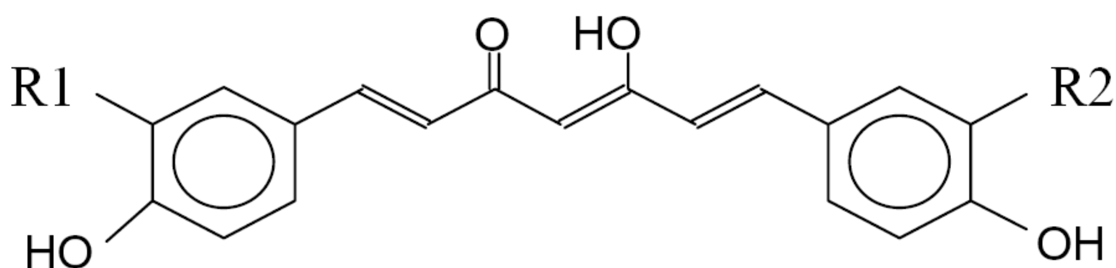
As indicated earlier, turmeric contains three different analogues of curcumin. The chemical names and properties are shown below (Litwinienko and Ingold, 2004; Scotter, 2009):

Curcumin I: 1,7-bis-(4-hydroxy-3-methoxyphenyl)-hepta-1,6- diene-3,5-dione.
Chemical formula: $C_{21}H_{20}O_6$; Molecular weight: 368 g/mol. pKa= 8.54

Curcumin II: 1-(4-hydroxy-3-methoxyphenyl)-7-(4-hydroxyphenyl)-hepta-1,6-diene-3,5,-dione.
Chemical formula: $C_{20}H_{18}O_5$; Molecular weight: 338 g/mol. pKa= 9.30

Curcumin III: 1,7-bis-(4-hydroxyphenyl)-hepta-1,6-diene-3,5-dione.
Chemical formula: $C_{19}H_{16}O_4$; Molecular weight: 308 g/mol. pKa= 10.69

These compounds are practically insoluble in water at acidic and neutral pH, and soluble in methanol, ethanol, dimethylsulfoxide, and acetone. The maximum absorption (λ_{max}) of curcumin in methanol occurs at 430 nm (Goel et al., 2008).



Curcumin I : R1 = R2 = OCH_3

Curcumin II: R1 = OCH_3, R2 = H

Curcumin III : R1 = R2 = H

Figure 4: Chemical structures of curcuminoids (Aggarwal et al., 2007).

Molecular configuration of curcumin can exist in tautomeric forms, bis-keto and enolate. In acidic, neutral conditions and in solid phase, the keto form predominates, and curcumin acts as a potent donor of H-atoms. However, under alkaline conditions the enolic form predominates, as shown in Figure 5 (Strimpakos and Shrama, 2008).

Several researchers have proven the sensitivity of curcumin to light, and as a result they suggested that biologic samples containing curcumin should be protected from light (Strimpakos and Sharma, 2008). Another stability issue is the stability of curcumin in phosphate buffer. It was reported that most of curcumin (>90%) is rapidly degraded within 30 min of placement in phosphate buffer systems of pH 7.2 (Goel et al., 2008).

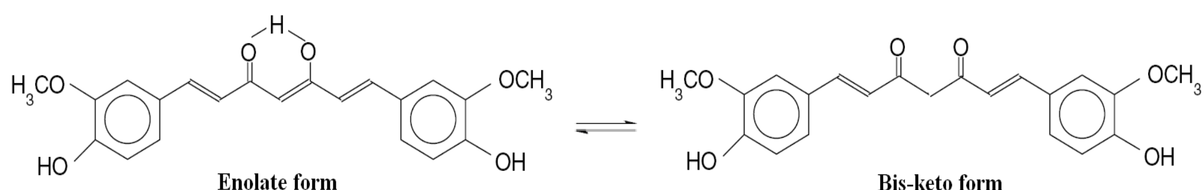


Figure 5: Keto-enol tautomerism of curcumin (Strimpakos and Sharma, 2008).

1.2.3. Pharmacological Effects of Curcumin Related to Inflammation

The accumulated evidences over the years have shown that many of anti-inflammatory drugs, such as steroids and NSAIDs are associated with numerous of side effects. Probably the best example is the cardiovascular complications caused by the use of most coxibs (Moodley, 2008). Consequently, there is an increasing demand for safer and more efficient anti-inflammatory agents. Curcumin has been reported as one of the most promising candidates of natural origin anti-inflammatory agents, with almost no reported side effects (Aggarwal and Sung, 2009).

Curcumin has been traditionally used in prevention and treatment of several conditions and diseases. Several of these effects have been already well documented scientifically. Studies indicated that curcumin exerts hepato- and nephro-protective, thrombosis suppressing, myocardial infarction-protective properties. Additionally, its strong antioxidant, antimicrobial, anticarcinogenic and anti-inflammatory activities were also reported (Aggarwal and Harikumar, 2009).

Until recently, many of the anti-inflammatory molecular targets of curcumin were unknown. However, the establishment of modern biology in the recent decades led to the discovery of more than 90 targets (Aggarwal et al., 2007). The mechanisms implicated in the anti-inflammatory potential of curcumin may include (Brouet and Ohshima, 1995; Kawamori et al., 1999; Aggarwal et al., 2007; Menon and Sudheer, 2007; Jurenka, 2009):

1) Suppression of the activation of the transcription factor NF–κB, which regulates the expression of pro-inflammatory gene products.
2) Down-regulation of the expression of cyclooxygenase-2 (COX-2), an enzyme linked with most types of inflammations.
3) Decreasing the activity and protein levels of inducible nitric oxide synthase (iNOS) enzymes through reducing the expression of iNOS genes.
4) Inhibition of arachidonic acid metabolism via lipoxygenase and scavenging the free radicals generated in this pathway.

5) Down-regulation of the expression of various cell surface adhesion molecules that have been linked with inflammation.
6) Decreasing the expression of various inflammatory cytokines, including TNF, IL-1, IL-6, IL-8, and chemokines.
7) Curcumin is a potent antioxidant, which contributes to its anti-inflammatory action.

All these effects are thought to lead to lowering the formation of inflammatory compounds and suppressing the inflammatory response. This outcome is considered to be beneficial in many abnormal conditions such as autoimmune diseases (Jagetia and Aggarwal, 2007). Furthermore, there are growing evidences linking many of the targets mentioned above with tumor promotion (Suresh and Srinivasan, 2007; Jurenka, 2009). Studies have shown that enzymes such as COX-2 and iNOS overexpression have been implicated in the carcinogenesis of many tumors (Brouet and Ohshima, 1995; Surh et al., 2001; Menon and Sudheer, 2007). Although it has not a direct effect on the human cells, it should be noted that antimicrobial activity of curcumin is potentially chemopreventive because an increasing evidence that number of pathogens are directly linked with human cancers (Strimpakos and Sharma, 2008).

1.2.4. Limitations in Formulating Dosage Forms and Delivery Systems

Despite the demonstrated efficacy of curcumin, it appears that its poor systemic bioavailability after oral dosing compromises the potential for therapeutic uses. The major reasons contributing to the low bioavailability of curcumin include poor absorption and rapid systemic elimination (Strimpakos and Sharma, 2008).

Oral drug administration is usually considered as a practical and easy way to administrate drugs. However, in order for a drug from solid dosage form to be absorbed, in this case through the epithelial layer of the intestine, these substances must become dissolved. Curcumin is a hydrophobic compound with very low solubility in water. The partition coefficient and solubility in water was measured to be 3.2 and 0.6 µg/ml, respectively (Kurien et al., 2007; Patel et al., 2009). When water-solubility is less than 1 µg/ml, which is the case for curcumin, the bioavailability from oral formulations such as conventional

tablets may be unacceptable (Pouton, 2006). This was demonstrated in clinical trial study to evaluate the pharmacokinetics and effective dose of curcumin in humans. In this study a number of patients were given 8000 mg of free curcumin orally per day in order to achieve detectable systemic levels. However, beyond 8 grams, the bulky volume of the drug was unacceptable to the patients (Cheng et al., 2001; Bisht et al., 2007).

Furthermore, studies performed on humans and animals shown that orally administrated curcumin undergoes rapid metabolism in the liver particularly via glucuronidation, while curcumin given intraperitoneally or systemically undergoes reduction (Aggarwal and Sung, 2009). Metabolites produced from these pathways show low or no pharmacological activity (Aggarwal et al., 2007; Aggarwal and Harikumar, 2009).

1.2.5. Delivery Systems for Curcumin

It is necessary to improve the bioavailability of curcumin in order to fully utilize the potential of this agent, and therefore a growing number of research groups are working on this aim. There are studies designed to investigate new approaches that could overcome these limitations seen with free curcumin. Number of studies has evaluated the liposomal formulation *in vivo* and their effectiveness. The study conducted by Li et al. (2005) investigated the effect of liposomal curcumin on pancreatic carcinoma cells and suppression of KF-kB activity. The incorporated curcumin in liposomes showed a dose-related increase in apoptosis of carcinoma cells and suppression of NF-κB activity. Moreover, the liposomal curcumin was found to be as effective as or better than free curcumin. Another experiment studied the effect unilamellar liposomal curcumin after tumor implantation on mice. It concluded that liposomal curcumin could increase the life span of the animals by up to 74% in comparison with untreated (Rubya et al., 1995). The study conducted by Kunwar et al. (2006) compared the cellular uptake of liposomal and albumin-loaded-curcumin by the spleenic lymphocytes and EL4 lymphoma cells. They reported that liposomes were able to deliver more curcumin into the cells than human serum albumin.

The absorption of a micellular formulation was evaluated using everted rat intestinal sacs. This micellular formulation was composed of phosphatidylcholine and sodium deoxycholate. The authors reported that after the incubation for 3 hours, the percentage of free curcumin absorbed was 49%, whereas the percentage for micellular formulation was 56%. (Suresh and Srinivasan, 2007).

In another approach phospholipid complex of soya phospholipid and curcumin was tested *in vivo* on rats. The study showed higher plasma concentrations, and longer half-life of phospholipid complex in comparison with free curcumin. Furthermore the bioavailability was also seen to be improved significantly after oral administration. The relative bioavailability of curcumin was estimated to be around 330% for the phospholipid complex as compared to free curcumin (Liu et al., 2006).

Another strategy of delivering curcumin is self-microemulsifying drug delivery system (SMEDDS). This system is basically composed of isotropic mixtures of oil, surfactant, co-surfactant and drug which has the ability to form o/w microemulsion when it comes in contact with aqueous medium in gastro intestinal tract after oral intake (Borhade et al., 2008). The curcumin-SMEDDS formulation was composed of 57.5% surfactant, 30% co-surfactant and 12.5% oil. The *in situ* evaluation of this formulation showed that the absorption percentage of curcumin-loaded SMEDDS was 3.86 times higher than that of curcumin suspension (Cui et al., 2009).

“Nanocurcumin” is another formulation recently developed for curcumin. The principle of this formulation is that curcumin is encapsulated in cross-linked polymeric particle with a hydrophobic core and a hydrophilic shell. The size of these particles lies in nanometer range and typically less than 100 nm. The group tested the product on pancreatic cancer cells and NFkB and reported to be effective in inhibition of these cells and has similar activity as free curcumin on inflammatory cytokines (Bisht et al., 2007).

Loaded solid lipid nanoparticles (SLN) is another type of nano-particle based delivery formulations. The system is usually consisting of biodegradable solid lipids. At room temperature the particles are in the solid state. Therefore, the mobility of incorporated molecules is reduced, thus it may offer possibility of modified release (Mühlen et al., 1998). The study on SLN loaded curcumin was preformed by Tiyaboonchai et al. (2007)

and aimed at using this formulation in topical application. The stability and release was tested and found that properties of cream containing curcumin incorporated into SLNs was improved in comparison to free curcumin in the cream formulation.

Using pharmacological agents such as piperine (a component of black pepper) as suppressor of glucuronidation process of curcumin was also investigated. It was reported the inhibition of this process which occur primarily in the liver and in the intestine could enhance the bioavailability of curcumin (Aggarwal et al., 2007).

As presented, there are numerous studies suggesting different approaches of delivery systems in order to improve the absorption of curcumin. All of these studies have concluded that it is possible to develop formulations and methods which can improve the bioavailability and give higher plasma concentrations.

1.3. Liposomes as Drug Delivery System

A liposome is defined as a self forming structure consisting of one or more concentric spheres of lipid bilayers separated by water or aqueous buffer compartments (Lieberman et al., 1998). Phospholipids are the backbone of these structures. Phosphatidylcholine (PC), also called lecithin, is a biocompatible phospholipid that exists in plants and animals and used frequently in liposomal preparation. Moreover, there are other molecules widely used in combination with phospholipids, such as cholesterol (Weiner et al., 1989; Torchilin and Weissig, 2003). The exact location of a drug in liposomes will depend upon its physicochemical characteristics and the composition of the lipids (Weiner et al., 1989). However, as a general rule, the hydrophilic drug molecules can be encapsulated in the aqueous space whereas the hydrophobic and amphiphilic molecules can be incorporated into the lipid bilayer, as presented in Figure 6 (Hupfeld et al., 2006).

Numerous evidences have demonstrated the ability of liposomes to enhance the efficiency of drug delivery via several routes of administration (Egbaria and Weiner, 1990). One of the major effects of liposomes as drug carriers is altering the pharmacokinetics of drug. It is known that pharmacological response is dependent upon the concentration of the drug in

the target cell. The drug concentration in the target site is governed by absorption, distribution and elimination. These processes may influence the pharmacokinetics of the drug and lead to inefficient utilization of the therapeutic agent. Thus, higher doses need to be administrated. Furthermore, higher drug doses often lead to resistance and undesirable immunological and toxicological effects (Fendler and Romero, 1977).

Liposomes are thought to shield all or most of the drug molecules resulting in decreasing the direct contact of drug with biological environment, thus the pharmacokinetic profile of the drug will be determined by the physiochemical properties of liposomes, rather than the drug itself (Sætern, 2004). Incorporating the drug into a vehicle capable of delivering it intact would overcome many of the disadvantages of the free drug administration. Improving the pharmacokinetics of the drug by this method could lead to beneficial effects such as reduced dosages, increased cellular permeability and delayed drug elimination (Fendler and Romero, 1977). It is worth mentioning that liposomes are also non-toxic, biodegradable and can be manufactured on large scales (Washington et al., 2001).

The potentials of liposomes to serve as delivery systems have been proven by the number of liposomal formulations already approved by FDA for clinical use such as AmBisome® (amphothericin B) and DaunoXome® (Daunorubicin). These formulations were clinically compared with conventional drug formulations and proved superiority of liposomal delivery (Allison, 2007).

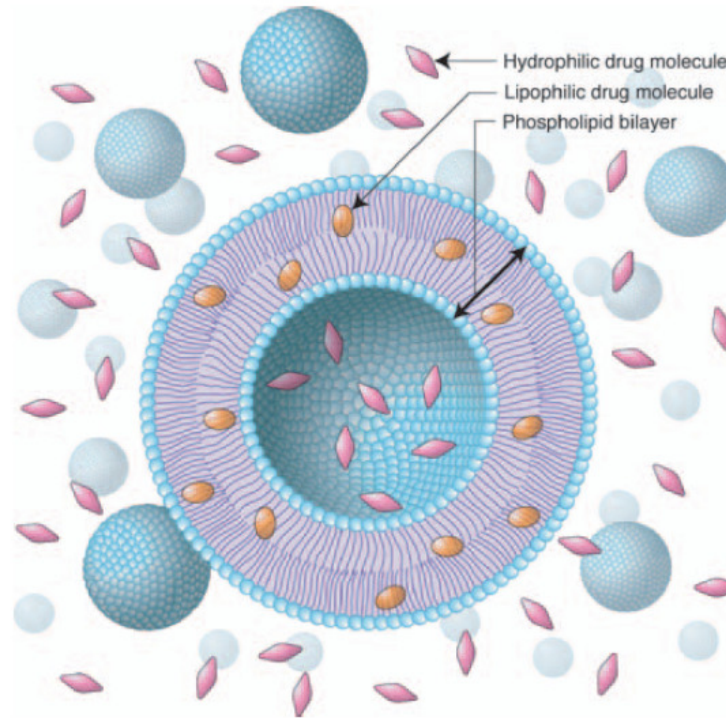


Figure 6: Schematic illustration of small unilamellar liposome-drug carrier with hydrophilic drug in the aqueous compartments and lipophilic drug incorporated into the phospholipid bilayer (Hupfeld et al., 2006).

1.3.1. Classification of Liposomes

Liposomes are usually classified according to their lamellarity and size. The following categories show the major types of liposomes (New, 1990; Philippot and Schuber, 1995):

Multilamellar vesicles (MLV): This population has a broad range of size distribution that occurs in a range of 100-1000 nm. The lipid composition may influence the lamellarity of these MLVs. However, the lamellarity typically varies between 5 and 20 concentric lamellae.

Large unilamellar vesicles (LUV): The size of these vesicles is normally up to 1000 nm and the structure consists of a single lamellae.

Small unilamellar vesicles (SUV): The structure normally consists of single lamellae and the diameter of this population is below 100 nm.

The choice of preparation method can influence the size and lamellarity of liposomes, resulting in different characteristics and fate of entrapped drug *in vivo* (Philippot and Schuber, 1995).

1.3.2. Methods of Preparation

There are various methods to produce different types of liposomes. However, all preparation methods can be simplified as to involve three basic steps: 1) Preparation of the dispersion of the lipids in aqueous media 2) purification of prepared liposomes, and 3) analysis of final product (New, 1990). The following is a brief description of methods that are considered among the most widely used in liposome preparation:

Hand-shaking, (MLV)

The principle of this method is to dissolve the lipid in efficient solvent. Lipophilic drugs can be dissolved in compatible solvent which can be added later to the lipid solution. This mixture will be dried down under pressure till it forms lipid films. Hydrophilic drugs can be dissolved in aqueous solution which will be added to the lipid films under shaking. This will produce swelling and peeling of the lipid films and gives a milky suspension of MLVs. Using this technique, one can entrap as high as 100% of the lipid soluble molecules, whereas hydrophilic compounds are often encapsulated in amount of 5-10% (New, 1990).

Sonication, (SUV)

Sonication is the most widely used method for producing small vesicles. It is usually used to convert MLVs to SUVs through the employment of energy at high levels by exposure of MLVs to ultrasonic irradiation. Probe-sonication and bath sonication are frequently used techniques to reduce the size of liposomes. The probe has the most efficient transfer of energy to the liposomal dispersion. However, it is associated with metal particle shedding from its probe and therefore one must be aware of the potential contamination. Bath sonication is much milder than probe sonication, but it is time-consuming and may result in low yield of smaller liposomes (New, 1990).

Extrusion, (LUV)

The principle in extrusion technique is based on employment of moderate pressure to force MLVs through polycarbonate filters with defined pore size. At low pressures (100 psi), MLVs display a reduced-size while maintaining their multi-lamillarity, whereas at higher pressure the liposomes are broken down as they pass though the membrane filter resulting in reorganizing of the phospholipid bilayer giving rise to unilamellar vesicles (Philippot and Schuber, 1995). It is simple method and easy to use and there are several products available on the market. Among the equipments are the Hamilton® syringes. These are two syringes connected by a filter holder allowing the samples to pass back-and-forth through the polycarbonate filter. However, this method can be limited by production of small volumes of LUVs and the back pressure that can be tolerated by the syringe and filter holder is limited (Gregoriadis, 2007).

Unprocessed MLVs have limited uses in *in vivo* studies because of their large diameter and heterogeneity of size (Gregoriadis, 2007). However, the techniques used to change these parameters may influence physical properties of liposomes. The conversion to SUVs from MLVs may result in vesicles with very low trapped volumes. Furthermore, SUVs can be unstable and prone to fusion process due to the high curvature of the lipid bilayer (New, 1990). Extrusion technique used to produce LUVs may result in rupturing and resealing which leads further to leakage of the entrapped drug and the final vesicles may have lower amount of entrapped material, depending on the lipophilicity of the drug (Gregoriadis, 2007).

1.3.3. Topical Administration

Liposomes have been widely used to enhance the efficiency of drug delivery though various routes of administration and have been shown to be significantly superior to conventional dosage forms especially for intravenous and topical administration (Egbaria and Weiner, 1990). However, therapeutic applications of systemically administered liposomes have been limited by their rapid clearance from the bloodstream and their uptake by reticuloendothelial system (RES) in liver and spleen. Furthermore, the use of liposomal formulations in oral administration has been limited due to physiological factors.

The three major factors are pH, bile salt, and pancreatic enzymes in the gastrointestinal (GI) tract can destabilize the structure of the vesicles and limit their potential (Lian and Ho, 2001).

Topical liposomal administration might offer an opportunity for developing a novel delivery system that could overcome these limitations experienced with the systemic and oral liposomal formulation as well as conventional products. The major advantages of topical liposomal drug include (Egbaria and Weiner, 1990):

1) Reduction of side effects and incompatibilities that may arise from undesirably high systemic absorption of drug.
2) Markedly increasing the liposomal drug accumulation in the desired tissues.
3) Capability for incorporation of a wide variety of hydrophilic and hydrophobic drugs.

Additionally, their ability to provide a sustained/controlled release and an enhancement of the cellular penetration of the incorporated material could improve their potential for being applied vaginally (Pavelic et al., 1999; Pavelic et al., 2004b).

1.3.4. Vaginal Application

Liposomes due to their ability to encapsulate both lipophilic and hydrophilic active ingredient, represent a promising delivery system in regard to vaginal therapy. Some of studies have investigated the release profiles of number of pharmaceutical agents incorporated in liposomal formulations in simulated vaginal conditions. The study conducted by Pavelic et al. (1999) had an aim to investigate the stability of liposomal formulations for metronidazole, clotrimazole and chloramphenicol, drugs which are frequently applied in the treatment of vaginal infections. The group concluded that the liposomes retained approximately 28-40% of the entrapped drug even after 6 hours of incubation in an environment that mimics the vaginal cavity of pre- and post postmenopausal women.

The cervical mucus present in the vagina is believed to assist in the bioadhesion process (Brannon-Peppas, 1993). However, being in a liquid form as liposomal suspensions, there might be higher probability for the formulation to be expelled from the vagina and reduce

the retention time at the site of action (Pavelic et al., 2001). Therefore research groups are considering incorporation of liposomes in a bioadhesive base, such as hydrogels.

Among various types of hydrogels, Carbopol hydrogels have demonstrated a good compatibility with liposomal formulations (Skalko et al., 1998). The polymer adhesion to tissues permits intimacy of contact and also improves the drug absorption. Furthermore, it also prolongs the residence time at the site of administration (Knuth et al., 1993).

One study investigated the comparison of the release profiles between liposomal gels containing hydrophilic marker substance (FITC-dextrans) with the same gel with the marker present in non-liposomal form. The conditions in this study were set to be close to the physiological environment. The retention of encapsulated hydrophilic model compound FITC-dextrans was much higher than the control gel without incorporated liposomes. It was found that about 20% of dye was released from liposomes incorporated in the gel after 72 hours, whereas the control gel released approximately 89% during the same period (Pavelic et al., 2004b). Another study was investigating the effect of Carbopol gel on the stability of liposomal chloramphenicol. It was calculated that more than 40% of the entrapped drug was in the liposomal gel even after 24 hours of incubation in simulated vaginal conditions. The authors concluded that the gel formulation provided a stable vehicle suitable for vaginal application in which liposomes are distributed uniformly and their original size distribution of liposomes is preserved (Pavelic et al., 2004a).

The same group compared the *in vitro* stability of liposomal suspensions and liposomal Carbopol gel formulations of acyclovir, an antiviral agent with low bioavailability especially in topical dosage forms. Acyclovir was encapsulated in three types of liposomes, namely neutral, negatively- and positively- charged liposomes. However, even with incorporation of different liposomes in hydrogels the results showed the slower release profile of drug from liposomal gel in comparison with liposomal suspension, as well as protective effect of a hydrogel matrix on liposomes which yielded in improvement of stability (Pavelic et al., 2005).

2. AIM OF THE STUDY

The aim of this project has been to develop liposomal formulation for curcumin in order to improve its solubility. Liposomes would serve as carrier system enabling the highly lipophilic substance to be prepared in aqueous formulation. For that purpose liposomal preparations were optimized in order to:

- Study the size and methods of size reduction for liposomes containing curcumin.
- Measure the entrapment efficiency in prepared formulation and optimize the preparation method.
- Investigate the stability of liposomal formulations using accelerated stability testing.
- Evaluate the curcumin potential as an anti-inflammatory agent by using DPPH assay.

3. METERIALS AND METHODS

3.1. Materials

3.1.1. Chemicals

Table 1: Chemicals

Chemical	Manufacturer / provider
Calcium chloride hexahydrate	AnalaR, UK
Cholesterol (lanolin)	Sigma Aldrich, USA
Curcumin	Sigma Aldrich, India
Chloroform	Merck, Germany
Ethanol 96%	Acrus, Norway
Ethyl acetate	Merck, Germany
Lipoid S100	Lipoid, Germany
Methanol (LiChrosolv)	Merck, Germany
Sepharose CL-4B	Pharmacia Biotech, Sweden
Tris-HCl (Tris(hydroxymethyl)aminomethane-hydrochloride)	Merck, Germany
Triton X-100	Merck, Germany

3.1.2. Solutions

The following solutions are given in examples of 1L volume:

Triton 2.5% Buffer pH 8.0

- Used to prepare standard solutions for measurements of lipid content in supernatant and pallets in the evolution of the separation method for formulations which dose not contain cholesterol.
  1. Calcium chloride hexahydrate 0.075 g
  2. TRIS-HCl 7.88 g
  3. Triton X-100 26.74 g
  4. Adjust to pH 8.0 by adding 1 M NaOH q.s.
  5. Distilled water ad 1000 mL

Triton 10% Buffer pH 8.0

- Used to prepare standard solutions for measurements of lipid content for the evaluation of the separation method for cholesterol containing formulations.
  1. Calcium chloride hexahydrate 0.075 g
  2. TRIS-HCl 7.88 g
  3. Triton X-100 107 g
  4. Adjust to pH 8.0 by adding 1 M NaOH q.s.
  5. Distilled water ad 1000 mL

3.2. Methods

3.2.1. Preparation of Liposomes

Preparation of lipid solutions:

PC solution: A solution of soya phosphatidylcholine (PC; Lipoid S100) was prepared by dissolving 200 mg of PC in 5 mL methanol.

PC/Chol 4:1 solution: An amount of 25.4 mg of cholesterol was dissolved in 5 mL methanol/chloroform with (4:1; vol/vol). Then 200 mg of PC was added to the solution to obtain the molar ratio of 4:1.

PC/Chol 2:1 solution: The same procedure as PC/Chol 4:1 solution was used to prepare this solution except the amount of cholesterol used was 50.9 mg.

Table 2: Composition of lipid solutions.

Formulations	Molar Ratio	Amount of Lipids		Solvent
		Phosphatidyl choline (mg)	**Cholesterol (mg)**	
PC	1	200	-	Methanol
PC/Chol	4:1	200	25.4	Methanol/Chloroform (4:1; vol/vol)
PC/Chol	2:1	200	50.9	Methanol/Chloroform (4:1; vol/vol)

Preparation of Curcuma extract

Curcuma powder (100 g) was extracted with 1) water (2000 mL), 2) 96% Ethanol (2000 mL) and 3) Ethyl acetate (200 mL), respectively. For each extraction the mixture was bath sonicated for 10 minutes and left overnight with occasional shaking prior to filtration. The residues were separated after filtration and solvents in the filtrate evaporated with rotary

evaporator at low pressure. Percentage yield was expressed as w/w of dry powder from the market.

Preparation of lipid film by rotary evaporation and hydration

The dissolved lipids (5 mL) were transferred into three separate round bottom flasks of 500 mL. In the case of curcumin containing liposomes, the drug was dissolved in methanol yielding a concentration of 5 mg/mL (extract or curcumin mixture) or 1 mg/mL (curcumin I) and mixed with lipid solutions. The solvent was removed under vacuum of 55 hPa and rotation of 60 rpm at 25 °C in 90 minutes by a Büshi R-124 rotary evaporator with vacuum pump 500-system (Büshi, Switzerland). The deposited lipid film was removed from the rotary evaporator and left at room temperature for an additional period of 60 minutes to remove traces of solvent. Subsequently the lipid film was hydrated using 10 mL of freshly distilled water. The resultant liposomal suspension was manually shaken for 15 minutes till homogeneous suspension was obtained. The suspension was left at room temperature over night prior to further treatments.

3.2.2. Size Reduction of Liposomes

Sonication

Liposomal suspension of MLVs (1.8 mL) transferred to a 2 mL round bottom vial (Eppendorf, Germany) and placed in ice bath. The position of a needle probe tip 407 probe-sonicator Labsonic U (B. Braun Biotech, Germany) was fixed in vial. Vertically, it was fully immersed into the vial and horizontally, it was positioned in the middle of the volume. The liposomal suspension was exposed to ultrasonic irradiation with an output of 30, 40 and 50 Watt and duration of continuous 30 and 150 seconds, as described in Table 3. The sample was left to cool down and placed in the fridge at 4 °C for 1 day prior to further test e.g. size analysis and centrifugation. This experiment was executed in order to determine suitable sonication procedure. After comparing the results a final method for sonication was chosen to be 40 W for 150 sec.

Table 3: Preliminarily experiments to investigate sonication variables in order to determine their influence on size and size distribution.

Output (Watt)	Duration (Seconds)	
	30	**150**
30	Empty PC, PC/Chol (2:1)	Empty PC, PC/Chol (2:1)
40	Empty PC, PC/Chol (2:1)	Empty PC, PC/Chol (2:1)
50	Empty PC, PC/Chol (2:1)	Empty PC, PC/Chol (2:1)

For each sonication conditions the experiment was performed in duplicate.

Extrusion

The liposomal suspension was filter-extruded through a polycarbonate membrane Track-Etch Nuclepore membrane (Whatman, UK). Up to 1 mL of MLVs were passed five times, back-and-forth, through the 0.4 µm polycarbonate membrane filters at room temperature. The extrusion was done by hand with a syringe extruder Liposofast™ (Avestin Inc., Canada). The resultant products were stored in the fridge at 4 °C over night prior to size analyses and centrifugation.

3.2.3. Separation of Unentrapped Active Ingredients

Centrifugation

For separation of liposomes from unentraped active ingredient, a portion of the liposomal dispersion was transferred to 3-mL thick wall polycarbonate centrifuge tubes. The samples were then centrifuged using SW60Ti rotor and Beckman Optima L8-M centrifugator (Beckman Inc., USA). The centrifugation was done at a temperature of 10 °C and speed of

35000 rpm (150000 g) for 150 minutes. The content of active ingredient in both supernatant and pellet was determined.

Size Exclusion Chromatography

Suspension of liposomes was fractionated by size exclusion chromatography as well. A MLVs sample of PC/Chol (molar ratio 2:1) formulation containing 5 mg curcumin was exposed for mild ultra-sonic irradiation in bath-sonicator for 5 minutes and vortexed for 1 minute prior to the separation. The sample was then added to a glass tube packed with Sepharose CL-4B (Pharmacia Biotech, Sweden) to approximately 25 mL (25 cm). Distilled water was added repeatedly to insure continues flow and prevent the column from drying. The collection of the portions was started immediately and continued until 6 samples after yellow color disappeared from the column, in total 30 mL including the void volume. The flow rate was estimated to be 0.5 $mL \cdot min^{-1}$ and took about 60 minutes for a sample of 1 mL of PC/Chol (2:1) liposomal formulation to pass through.

3.2.4. Particle Size and Size Distribution Analysis

Photon correlation spectroscopy (PCS):

Also known as dynamic light scattering is a simple and rapid method to determine the particle size and size distribution of liposomes. The principle is based upon the Brownian motion of particles in medium. As the particles diffuse in the fluid the collisions with medium molecules causes a random movement of the particles. When the PCS machine focuses laser light on the sample, it registers the signals from the moving particles as fluctuations in the scattered light. The analysis is based on the time dependence of these fluctuations. Small particles diffuse more rapidly than larger ones, thus the fluctuations vary accordingly. The software then calculates the radius of the particles by using Stokes-Einstein equation (NICOMP, 1997; Torchilin and Weissig, 2003).

Experiment

PCS measurements were performed on NICOMP Submicron particle sizer, Model 370 (NICOMP Particle Sizing Systems, USA). Sample preparations were preformed in a laminar airflow bench. The cuvettes (borosilicate glass) were cleaned and filled with distilled water and sonicated in bath-sonicator for 30 minutes to reduce the possibility of contaminations. Before measurements were preformed, the instrument parameters were adjusted according to the values listed in Table 4. The samples were diluted with freshly filtrated distilled water using an Acrodisc 0.2 µm syringe filter (Pall Corp., USA) to obtain an intensity count rate between 250 and 350 kHz.

Table 4: PCS parameters.

Parameters	Values
Temperature	23 °C
Viscosity	0.933 cp
Liquid index of refraction	1.333
Intensity set point	300 ± 50
Channel width	Auto
Number of cycles	1 cycle
Run time	5 minutes

3.2.5. Determination of Entrapment Efficiency

The entrapment efficiency measurements were preformed on UV-spectrophotometer Aligent 8453 equipped with deuterium and tungsten lamp (Agilent Technologies, Germany). In order to quantify the content of curcumin in supernatant and pellets in samples, series of standard solutions were prepared. The known amounts of curcumin were dissolved in ethanol and diluted to obtain a stock solution of 1000 ng/mL. The standard solutions were then prepared using the stock solution the respective concentrations (100, 200, 400, 600, 800 and 1000 ng/mL). The absorbance was measured at 425 nm based on

the spectral analysis. A calibration curve of curcumin was developed by plotting absorbance versus concentration of standard solutions.

The supernatant and pellets were each dissolved in methanol. The measurements were done in triplicate. The entrapment efficiency was calculated using the following equation:

Equation 1:

$$\text{Entrapment efficiency (EE\%)} = \frac{A}{(A+B)} \times 100$$

Where A is amount of curcumin in pellet and B is amount of curcumin in supernatant.

3.2.6. HPLC Analysis of Standard Curcumin and Curcuma Extracts

High performance liquid chromatography (HPLC) (Waters, USA) system consists of a HPLC Water 2690 Separation Module, a Water 996 Photodiode Array detector. Column was YMC pro C_{18} (250 x 4.60 mm) joined with precolumn. Mobile phase: CH_3CN-2.5% acetic acid (54:46); Injection volume: 10 µL. The temperature of column was maintained 35 °C during the chromatoghaphic separation. The flow rate was 1.0 mL/min and run for 12 minutes. The eluting compounds were monitored at UV 425 nm.

Analysis of standard curcumin

Standard curcumin was evaluated for its purity as well as it was expected that standard curcumin contains the mixture of curcumin (curcumin I), desmethoxy curcumin (curcumin II) and bisdesmethoxycurcumin (curcumin III).

Analysis of Curcuma extracts

Water extract, ethanol extract and ethyl acetate extract were analyzed by the HPLC method as described above.

3.2.7. Phosphatidylcholine Quantification

Enzymatic phospholipid assay:

The principle of quantitative enzymatic phospholipid assay is based on a sequence of reactions preformed by three enzymes (Phospholipase D, Choline oxidase, Peroxidase) allowing the colorimetrical quantification of the lipid content. The chain starts with phospholipids (lecithin, lysolecithin and sphingomyelin) being hydrolyzed by phospholipase D and which results in choline to be released (Figure 7). The liberated choline acts as a substrate in a reaction preformed by choline oxidase which forms an amount of hydrogen peroxide. The latter takes part in a peroxidase-catalyzed coupling reaction resulting in red dye. The amount of choline is proportional to the amount of resulting quinoneimine, thus the amount of phospholipids contained in the sample can be determined by measuring the absorbance of the red color (Grohganz et al., 2003; BioMérieux sa, 2009).

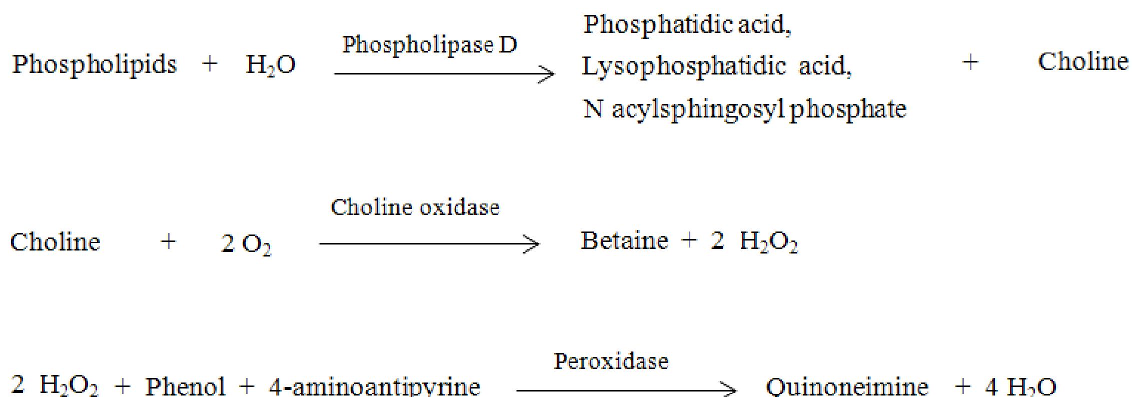


Figure 7: Schematic illustration of reactions in colorimetric determination of lipid content (BioMérieux sa, 2009)

<u>Experiment</u>

The amount of phospholipids was determined in terms of quantification of the phosphatidylchline content according to the protocol developed by Grohganz et al. (2003) and using an enzymatic kit, phospholipids enzymatique PAP 150 (BioMérieux sa, France) as a coloring reagent. The method can be divided into three stages namely, activation of

coloring reagent, preparation of standard solutions and finally measurement of the absorbance.

Activation of the coloring reagent

The preparation was done according to the user manual. The coloring reagent was activated by adding 25 mL of buffer solution (consisting of Tris pH 7.8, surfactant and phenol) to the dry enzyme reagent (Choline oxidase, Phospholipase D, Peroxidase and 4-aminoantipyrine).

Preparations of standard solutions

PC formulations: An amount of 100 mg of PC were dispersed in 10 mL of Triton 2.5% buffer in order to obtain a concentration of 10 mg/mL. The phospholipids in this mixture were not homogenously dissolved, and therefore it was necessary to use Retsch MM200 mixer mill (F. Kurt Retsch, Germany). To the mixture, 5 glass beads (diameter ~1 mm) were added and placed in mixer mill for 25 minutes at frequency of 30 Hertz. The mixture was warmed in a Memmert incubator (Memmert, Germany) for 16 hours to assure that all phospholipids are solubilized. After cooling down, 2 mL of 10 mg/mL stock solution was mixed with 18 mL Triton 2.5% buffer in order to get a concentration of 1000 µg/mL. This concentration was then used as a starting point for further dilutions. Six concentrations namely, 50, 100, 200, 400, 600 and 800 µg/mL were used in experiments.

PC/Chol (4:1) and PC/Chol (2:1) formulations: The same procedure was used as for PC formulations except that the amount of cholesterol and composition of Triton buffer were different. The amount of cholesterol used for PC/Chol (molar ratio 4:1) formulation was 12.7 mg, whereas the amount for PC/Chol (2:1) was 25.4 mg. The buffer used in these two measurements was Triton 10%.

Measurements of the absorbance

A volume of 50 µL for the standards and samples were mixed with 250 µL activated phospholipids reagent solution in a microplate Costar 96 plate (Corning Inc., USA). The plates were shaken for 5 minutes and incubated at 37 °C for 45 minutes. Some of the samples were diluted in order to get an absorbance within the standard curve range. The absorbance was measured with excitation filter A-492 nm on microplate reader PolarStar Galaxy (BMG Labtechnology, Germany). The measurements were executed in triplicates.

3.2.8. Stability Experiment

This experiment was preformed in order to predict the stability of the formulation using accelerated stability test (Florence and Attwood, 2006). The stability of empty and liposomes containing active ingredient were measured according to the method given below:

- Empty PC liposomes and liposomes containing curcumin, curcumin extract or curcumin, as well as empty PC/Chol (2:1) liposomes were sonicated at 40 W for 150 sec and left to cool down.
- Sonicated and untreated samples (MLVs) were centrifuged at the same conditions as decribed earlier.
- The quantity of curcumin was determined both in supernatant and pellet. The size of liposomes was measured in the PCS.
- All liposomal formulations were incubated at 40 °C in an incubator (Memmert, Germany) for 1 month period.
- Loss of entrapped curcumin (where applicable) and change in vesicle size were determined.

3.2.9. Determination of Antioxidant Activity of Curcumin and Curcuma Extract by DPPH assay

The radical scavenging (antioxidant) activity and the superoxide anion radical scavenging activity were determined as described by Basnet et al. (1997). The 1, 1- diphenyl-2-picryl hydrazyl (DPPH) (Sigma Aldrich, Germany) is a relatively stable free radical.

In brief, 1 mL of methanolic solution of each sample at various concentrations (10, 50 and 100 µg/mL) was mixed with 1 mL of methanolic solution of DPPH (approx. 60 µM). The reaction mixture was shaken vigorously and left for 30 min at room temperature.

The radical scavenging (antioxidative) activity of samples corresponding to the scavenging of DPPH radical was measured at 520 nm by absorbance of UV-spectrophotometer Aligent 8453 (Agilent Technologies, Germany) by following formula:

Equation 2:

$$\text{Radical scavenging activity (\%)} = \frac{(A - B)}{A} \times 100$$

Where A is the absorbance of the control and B is the absorbance of the sample. Control represents the test solution without sample. Throughout all the determinations, ascorbic acid was used as the positive control.

Calibration curve was plotted and effective concentration (EC50) value was calculated. The antioxidative activity was expressed by EC50. The EC50 value is defined as the concentration (µg/mL) of the sample required for 50% reduction of the DPPH radical absorbance.

Each value represented the mean of three readings. Statistical comparisons were made by Student's t-test.

4. RESULTS AND DISCUTIONS

4.1. Optimization of Liposomal Preparation Method

4.1.1. Sonication Procedure

It is well known that the sonication process may influence the size and size distribution of liposomes (Woodbury et al., 2006). In order determine the optimal conditions for sonication, it was necessary to preform number of trials and evaluate the impact of duration of sonication and energy output on the liposomal characteristics. Sonication parameters were evaluated in regard to vesicle size and size distribution by using PC and PC/Chol (2:1) liposomal compositions (Table 5).

Table 5: Selection of sonication conditions.

Output (Watt)	Liposomal composition	Duration (Seconds)			
		30		150	
		Mean size (nm ± SD)	P.I. (mean ± SD)	Mean size (nm± SD)	P.I. (mean ± SD)
30	PC	805 ± 145	0.63 ± 0.03	145 ± 28	0.34 ± 0.01
30	PC/Chol (2:1)	912 ± 0	0.58 ± 0.09	665 ± 270	0.37 ± 0.03
40	PC	447 ± 76	0.43 ± 0.02	97 ± 5	0.34 ± 0.01
40	PC/Chol (2:1)	909 ± 4	0.39 ± 0.05	237 ± 122	0.37 ± 0.02
50	PC	236 ± 30	0.42 ± 0.02	162 ± 12	0.39 ± 0.04
50	PC/Chol (2:1)	489 ± 324	0.50 ± 0.02	199 ± 45	0.32 ± 0.01

The amount of phosphatidylcholine used was 200 mg. The results represent the mean of two separate experiments.

P.I. represents the polydispersity index used as indication of size distribution of vesicles. Lower values of P.I. indicate more homogeneous liposomal sample.

It is important to note that the PCS machine used for measurements was equipped with mono-modal (Gaussian) distribution and multimodal (NICOMP) distribution options. Both systems can give useful information about the size and size distribution in submicron

range. When the size distribution shows bimodal or tri modal vesicle population distributions (NICOMP), it is rather difficult to determine the actual mean diameter. The polydispersity index over 0.3 indicates that the vesicle populations are very polydispersed as can be seen by the SD as well (Table 5).
The size of the liposomes before the sonication was measured for all samples and found to have very high P.I. and Chi square (χ^2), which is used to describe the quality of the fit. If the χ^2 range between 0 and 2; the Gaussian can be used to determine the size. However, if the value is higher than 3 it seemed reasonable to choose the NICOMP system.
The measured size of the liposomes was found to be very large and beyond the submicron range. The upper size limit that can be displayed on the NICOMP distribution is usually given as 912 nm, although this is not necessary to the actual size of the liposomes but it could indicate that the samples contain very large particles (> 1µm) as well.

Two samples from different batches of PC formulations were tested. By keeping one parameter constant while changing the other, all the parameters presented in Table 5 were tested on both PC and PC/Chol (2:1) formulations. The test started with low energy output, in this case 30 W, and lasted for a short time e.g. 30 sec. The result obtained from this condition suggested that by operating with short durations, the particle size will be large and P.I. is high indicating that the efficiency of size reduction is low and the samples are still containing highly polydispersed population of liposomes. However, as the energy output increases, the size appears to decrease in both formulations. In the case of PC/Chol (2:1), the results show larger particle size with larger standard deviations in comparison with PC only formulation. The duration of the sonication process was increased from 30 to 150 seconds. The result showed an obvious decrease in size and P.I. suggesting that it is rather more efficient to prolong the sonication time to 150 seconds instead of 30 seconds. Furthermore, the difference in liposome size between PC and PC/Chol (2:1) formulations appears to decrease as the energy output increases.

The primary goal was to obtain a sufficient size reduction and more monodispersed liposomal size for both liposomal compositions. The targeted size of liposomes was set to be around 300 nm (Skalko et al., 1998). After comparing the results, it seemed that there are two conditions e.g. (40 W or 50 W for 150 sec) that could give desired size of liposomes. Although in the case of sonication with 50 W the difference in size between the

PC and PC/Chol (2:1) formulations appears to be relatively small, the batches sonicated with 50 W (150 sec) showed tendency to agglomerate upon standing. Moreover, having in mind that as the size of liposomes is reduced, the amount of drug in liposomes may be reduced as well (Gregoriadis, 2007), the conditions of sonication under 40 W for 150 sec were selected. These conditions were again tested in preparation PC/Chol (4:1) liposomes. The mean diameter was found to be 126 ± 20 nm (P.I. of 0.38 ± 0.02). This size of PC/Chol (4:1) was between PC and PC/Chol 2:1 (see Table 5) and confirmed that the conditions of 40 W for 150 seconds were indeed suitable for sonication. It is expected that the inclusion of cholesterol in liposomal membrane makes liposomes more rigid and more resistant to size reduction (New, 1990).

Table 6: The effect of the amount of curcumin taken into the liposome preparation on liposomal size.

Liposomal composition	Curcumin (mg)	Mean particle size (nm ± SD)		P.I. (mean ± SD)	
		Nonsonicated	Sonicated	Nonsonicated	Sonicated
PC	0	912*	98 ± 4	1.03 ± 0.70	0.35 ± 0.01
PC/Chol (4:1)	0	912*	112 ± 28	0.44 ± 0.07	0.37 ± 0.03
PC/Chol (2:1)	0	912*	174 ± 61	0.45 ± 0.21	0.36 ± 0.06
PC	5	912*	196 ± 88	0.48 ± 0.09	0.34 ± 0.02
PC/Chol (4:1)	5	912*	138 ± 36	0.48 ± 0.16	0.38 ± 0.03
PC/Chol (2:1)	5	912*	175 ± 43	0.48 ± 0.07	0.33 ± 0.01
PC	10	912*	209 ± 20	0.42 ± 0.03	0.37 ± 0.02
PC/Chol (4:1)	10	912*	203 ± 82	0.50 ± 0.10	0.38 ± 0.02
PC/Chol (2:1)	10	912*	177 ± 28	0.49 ± 0.10	0.35 ± 0.02

* The size was too large to be accurately determined on PCS. The sonication conditions applied were 150 sec at power of 40 W. The amount of phosphatidylcholine used was 200 mg. The values denote the mean of three separate sets of experiments ± SD.

For easier comparison, the same results are presented in the Figure 8, and it appears that the size of sonicated PC formulations increases as the amount of drug used in liposomal preparation increased in comparison with empty control liposomes. These results

correspond to previous finding by Takahashi et al. (2008). The group measured the size of liposomes from soybean lecithins with different amounts of curcumin and discovered that as the amount of the drug increases the size of vesicles increases as well.

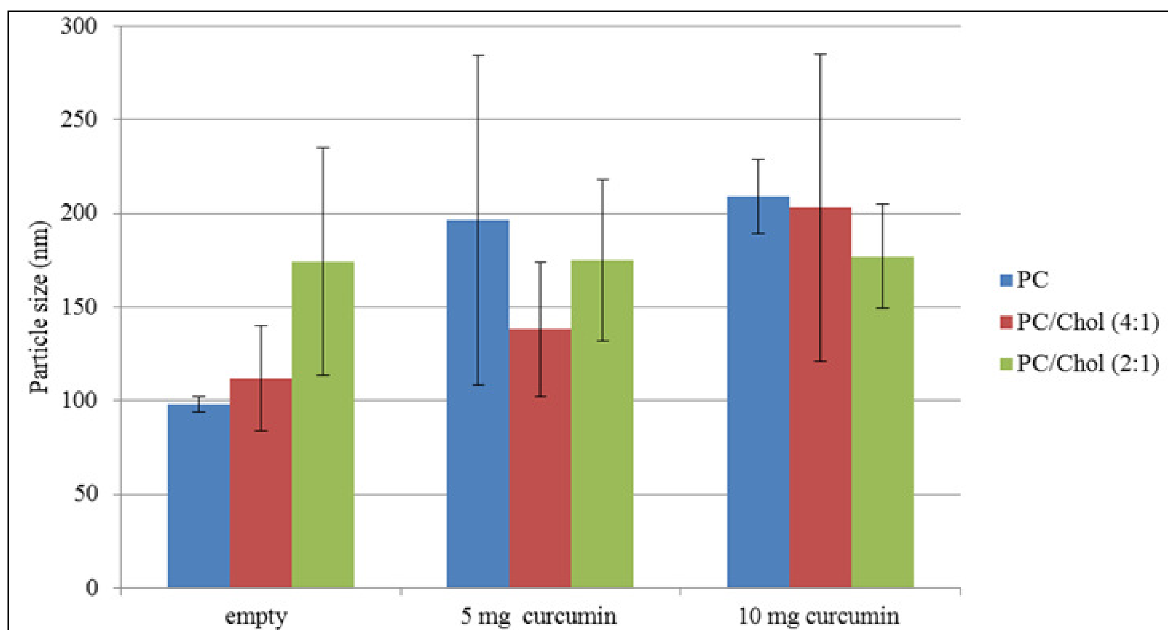


Figure 8: The effect of the presence of curcumin in liposomal membrane.

The sonication conditions applied were 150 sec at power of 40 W. The amount of phosphatidylcholine used was 200 mg. The values denote the mean of three separate sets of experiments ± SD.

The size of both PC and PC/Chol (4:1) liposomes containing curcumin appears to be larger than the size of empty liposomes. However, in the case of PC/Chol (2:1) liposomes, the size appears not to be affected by the presents of curcumin.

Regardless of the sonication conditions and type of liposomes, the standard deviation of the mean diameter and P.I. suggest that probe sonicator is not reducing the size in reproducible manner (Table 6).

4.1.2. Extrusion

Due to the problems with rather large SD measured for sonicated liposomes, we applied the extrusion process to reduce the particle size in more controllable manner.

Table 7: The effect of extrusion on liposomal size.

Liposomal composition	Curcumin (mg)	Mean particle size (nm ± SD)		P.I. (mean ± SD)	
		Nonextruded	**Extruded**	**Nonextruded**	**Extruded**
PC	0	912*	347 ± 21	1.03 ± 0.70	0.22 ± 0.02
PC/Chol (4:1)	0	912*	359 ± 39	0.44 ± 0.07	0.22 ± 0.01
PC/Chol (2:1)	0	912*	382 ± 8	0.45 ± 0.21	0.21 ± 0.02
PC	5	912*	310 ± 47	0.48 ± 0.08	0.22 ± 0.02
PC/Chol (4:1)	5	912*	362 ± 50	0.47 ± 0.16	0.15 ± 0.01
PC/Chol (2:1)	5	912*	408 ± 87	0.48 ± 0.07	0.21 ± 0.02

* The size was too large to be accurately determined on PCS.

The empty and liposomes containing 5 mg curcumin samples were extruded through a 0.4 µm polycarbonate membrane five times back-and-forth using Liposofast™ syringe at room temperature. The values denote the mean of three separte sets of experiments ± SD.

The size of extruded samples appears to correlate to the content of cholesterol. As the amount of cholesterol increases in the liposomes, the size of extruded liposomes increases as well. It is important to clarify that cholesterol dos not form liposomes by itself but it is known to cause an increase in the thickness of the lipid layer (Papahadjopoulos et al., 1972). This could probably be the reason for the cholesterol containing liposomes to show more residence to the reduction process, thus giving larger particles.

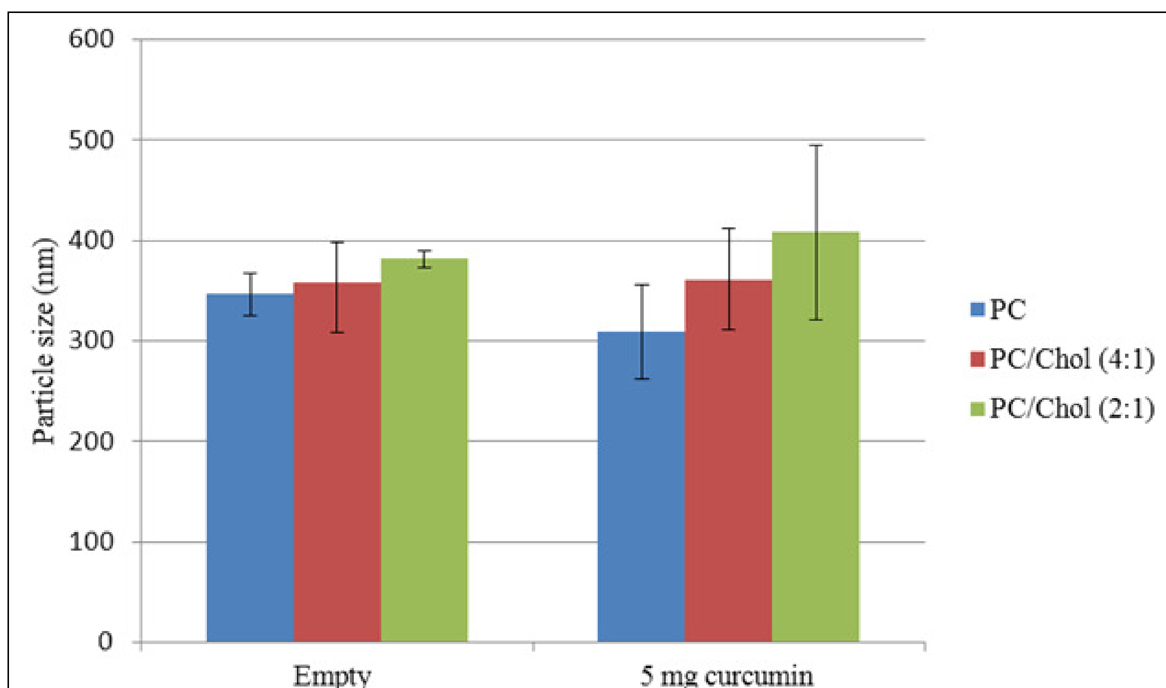


Figure 9: The size of extruded formulations.

The empty and liposomes containing 5 mg curcumin samples were extruded through a 0.4 µm polycarbonate membrane five times back-and-forth using Liposofast™ syringes at room temperature. The values denote the mean of three separte sets of experiments ± SD.

It appears that there is no size difference between the empty and liposomes containing 5 mg curcumin. This might be expected since the extruded samples typically have narrow particle size distribution, which is determined by the size of the pores they pass through (Thassu et al., 2007). However, during the extrusion process, an increase in resistance to the passage through the Liposofast™ syringes was noticeable when extruding the curcumin liposomes in comparison to the empty ones. Moreover, the samples with 10 mg of curcumin taken into the preparation were very difficult to extrude through the polycarbonate membrane, therefore it was necessary to preform the extrusion procedure only with empty and formulations containing 5 mg of curcumin. This might indicate that the size of the starting MLVs with 10 mg curcumin were larger or more tightly packed than empty or formulation with less curcumin, thus required more energy to perform the size reduction process.

Theoretically, it is possible to extrude samples first through larger pores and gradually decreases the size till the desired size is obtained. However, this process is not risk-free, in fact by doing so, there will be higher probability of the drug leaking out after each reduction step (Gregoriadis, 2007). Moreover this process can be very time consuming.

4.2. Entrapment Efficiency

4.2.1. Curcumin

The entrapment efficiency of curcumin in liposomes was calculated after centrifugation. In the case of untreated and extruded liposomes the amount of curcumin in the pellet medium was similar in all samples and ranged from 98-100%. Began et al. (1999) have reported that curcumin showed high binding affinity towards lecithin, apparently due to its hydrophobic nature.

In comparison with literature, Patel et al. (2009) and Takahashi et al. (2007) as well reported an entrapment efficiency of curcumin in liposomes of various compositions to be as high as 90% and 85%, respectively. However, a direct comparison with these values is difficult to make due to differences in experimental approaches.

In the case of sonicated samples, it appears that the entrapment efficiency increased as the amount of the added curcumin increased. Moreover, it was also observed that the PC/Chol (2:1) liposomes had the highest amount of the active ingredient whereas PC only liposomes had the lowest. The relationship between the amount of lipid used for preparation and the drug incorporated in the liposomes was investigated by Takahashi et al. (2009) and reported that as the content of lipid increases, the amount of curcumin incorporated in the liposomes increased as well. Similar tendency was also observed in our study. It appears that the entrapment of the drug is dependent on the composition of the liposomes and partly on the starting amount curcumin used in preparation of liposomes.

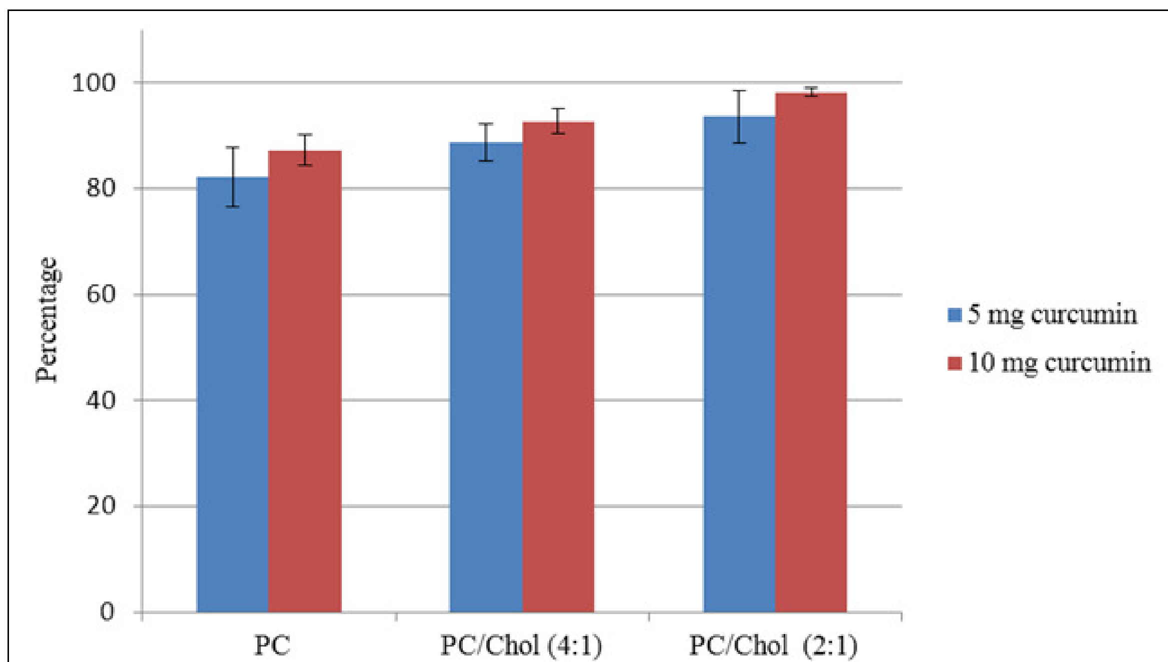


Figure 10: Entrapment efficiency for curcumin in sonicated liposomes.

The applied sonication conditions were 150 sec at output of 40 W. The samples were separated using centrifugation at 150000g for 150 minutes. The values are the mean of three separte sets of experiments ± SD.

It is important to note that the centrifugation conditions were extensive and expected to be sufficient to separate the pellets from supernatant in the sonicated formulations. The recovery of the entrapped drug varied between the formulations. In the case of PC only formulations, generally higher (over 90 %) curcumin recovery was observed whereas the PC/Chol (2:1) liposomes had the lowest recovery (80-90%). One explanation might be that the volume of methanol used to dissolve the liposomes was optimized for PC only formulations. This applies for liposomes prepared with lower amount of curcumin. However, it was difficult to obtain good recovery from the liposomal samples where 10 mg curcumin was used in preparation.

We tried to prepare liposomal preparations with curcumin by starting with 12.5 and 15 mg of curcumin, respectively. The visual examination of untreated 15 mg formulations revealed crystals sedimentation after centrifugation indicating unentrapped curcumin was precipitating out of dispersions, whereas the appearance of nonsonicated liposomes with

12.5 mg curcumin sample did not show any sediment formation in the first week after preparation. However, one month later at a temperature of 4 °C the same precipitate was observed and these preparations were not further tested.

The liposomal suspensions did not show any signs of precipitation before centrifugation and even the PCS measurements did not detect any unusual size distribution. However, the centrifugation probably induced loss of curcumin from the liposomes through the mechanical stress from this process which may have an impact on the drug present in the liposomes (Torchilin and Weissig, 2003). It would be advisable to use other separation methods, such as column chromatography. However, the process duration would than be much longer.

4.2.2. Curcuma Extract and Curcumin I

Curcuma Extract

The prepared Curcuma extracts yielded the following content expressed as the amount of curcumin present in the extract:

Water extract 11.3%
EtOH extract 18.3% and
Ethyl acetate extract 8.7%.
Percentage yield was expressed w/w of dry Curcuma powder from the market.

Curcumin I

Curcumin I was isolated from the standard curcumin as the UV spectroscopy, HPLC, NMR and Mass spectroscopy analyses data confirmed that the commercially available standard curcumin contains the mixture of curcumin (curcumin I), desmethoxy curcumin (curcumin II) and bisdesmethoxycurcumin (curcumin III).

The Curcuma extract and the pure curcumin I were incorporated into liposomes and the vesicle characterized as for preparations where standard curcumin was used, in corresponding amount of curcumin.

Table 8: Entrapment of Curcuma extract and pure curcumin in liposomes.

Material to be entrapped	Treatment	E.E. (%)	Size (nm)	P.I.
Extract	Nonsonicated	100**	911*	0.406
Curcumin I	Nonsonicated	100**	912*	0.666
Extract	Sonicated	81	103	0.353
Curcumin I	Sonicated	82	158	0.376

* The size was too large to be accurately determined on PCS; ** It was not possible to separate extract resides from the MLVs containing curcumin during the centrifugation.

The applied sonication conditions were 150 sec at output of 40 W. The samples were separated using centrifugation at 150 000g for 150 minutes. The values are the mean of three separte sets of experiments ± SD.

During the entrapment efficiency determination, the same volume of methanol was used to dissolve curcumin I as for the rest of the experiment. However, the solubility of curcumin I in methanol was low, and as a consequence the volume of methanol need to be increased from 1 to 5 mL.

The recovery of Curcuma extract and curcumin I appear to be very low (below 50%). However, as indicated earlier, the curcumin extract usually contains variety of substances which may interfere with the readings. In the case of curcumin I, the reason for the low recovery is unclear, but we suspect that it could be due to very limited solubility of curcumin I as compared to standard curcumin. Furthermore, we observed faster sedimentation of the curcumin I formulations both in the sonicated samples and MLVs. The reason for this phenomenon is unknown but this may influence the stability of the formulation and therefore further investigation is recommended.

As we observed that centrifugation has serious drawbacks in regard to separation of highly lipophilic natural origin substances from liposomes, we tried to evaluate the suitability of the separation method by determining the amount of phosphatidylcholine in both supernatants and pellets obtained after the centrifugation.

4.3. Evaluation of the Separation Method

4.3.1. Centrifugation

The relative content of phosphatidylcholine (PC) in the pellets as compared with whole dispersion revealed that nonsonicated formulations with 5 mg and 10 mg curcumin as well as extruded liposomal samples with 5 mg samples were separated sufficiently. There was no difference between the untreated and extruded formulations as all samples showed high recovery of phospholipids (>97%).

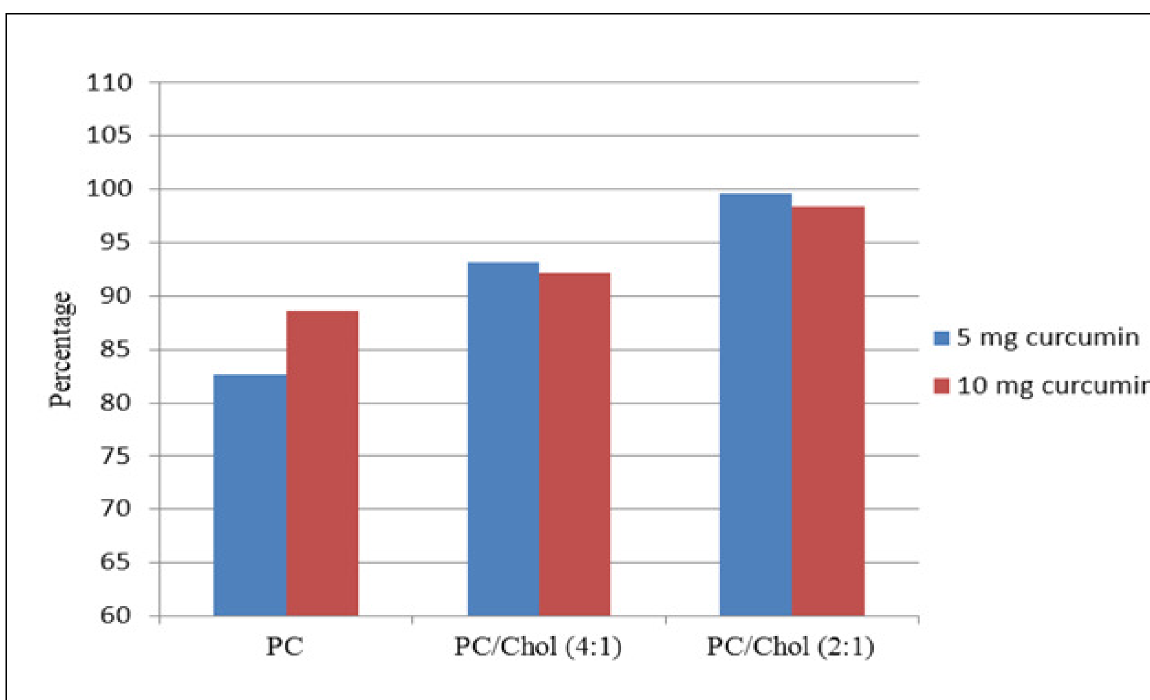


Figure 11: Phosphatidylcholine (PC) recovery from sonicated formulations.

The amount of phosphatidylcholine used for liposome preparation was 200 mg. Centrifugation was used as separation method (conditions 150000g for 150 min). The phosphatidylcholine was quantified by phospholipid assay and the relative amount of phosphatidylcholine calculated.

The relative percentage of recovered PC is similar to that of recovered curcumin in the supernatant as well as in the pellets. This similarity confirms that there is high entrapment efficiency of curcumin in the sonicated formulations.

The separation of liposomes from unentraped drug by using centrifugation is an easy and fast method. However, it appears that the chosen conditions in our experiment were not optimal. It was difficult to distinguish between supernatant and pellets for the sonicated formulations, especially when using pure Curcumin I and Curcuma extracts.

Cholesterol containing liposomes appear to be larger and denser than PC only liposomes and therefore the percentage of lipid recovered was higher (Figure 11). It thus appears that the addition of cholesterol to phospholipids modifies their molecular packing, which results in membranes which are more condensed than pure phospholipids (Papahadjopoulos et al., 1972).

We tried to evaluate another method of separation, column chromatography as well.

4.3.2. Size Exclusion Chromatography

This evaluation was preformed on PC/Chol (2:1) liposomes with 5 mg curcumin. The liposomes suspension of large MLVs was applied to gel column to separate any residual of free curcumin associated with the liposomes. The particle size of all fractions were measured in PCS. However only fractions 5-8 contained particles (Figure 12). The recovery from the fractions was calculated and found to be 70%. This low recovery is assumed to be due to sample loss during the fractionation. An explanation for this loss is probably that some agglomerated liposomes were too large to pass through the gel and remained on the top of the column. Nevertheless, the loss of curcumin in the column was calculated and amount of curcumin was normalized for this loss.

By this method the percentage of the free curcumin was calculated using Equation 3 and found to be 1.97% of the theoretical amount used in the test. In order to compare the results, a sample from the same batch was centrifuged and the amount of the free curcumin was calculated after normalizing for recovery and found to be 0.2%.

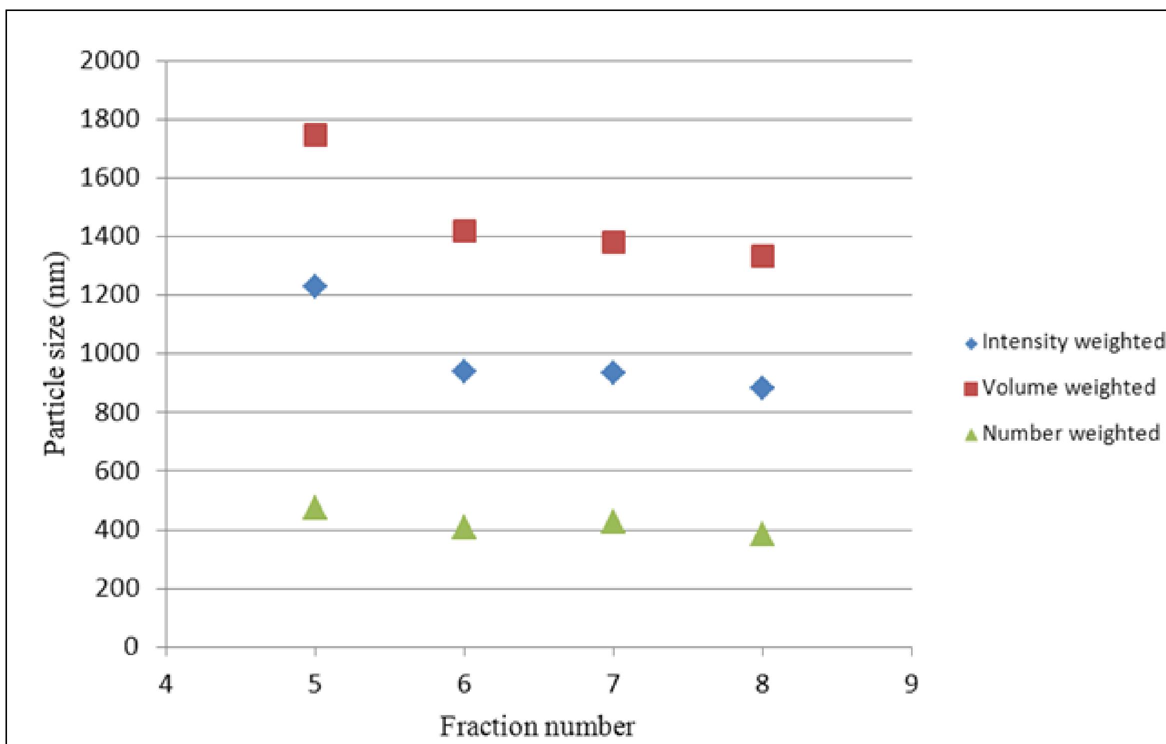


Figure 12: Size measurement of fractions collected during size exclusion chromatography (SEC) (Sepharose 4B-CL).

Equation 3:

$$\text{Percent of curcumin in fraction} = \frac{A}{B} \times 100$$

Where A is amount of curcumin in fraction #. And B is total theoretical amount.

Both methods of separation confirmed that there is high content of curcumin in the liposomes. Moreover this finding confirms that results obtained from the centrifugation are reliable, and therefore it was no need to use another separation method, especially one as time consuming as column chromatography. Moreover, the chromatography dilutes the liposomal samples as well.

Moreover, the results also implicate that the majority of curcumin is in the larger liposomes, and that the majority of formed liposomes are relatively large.

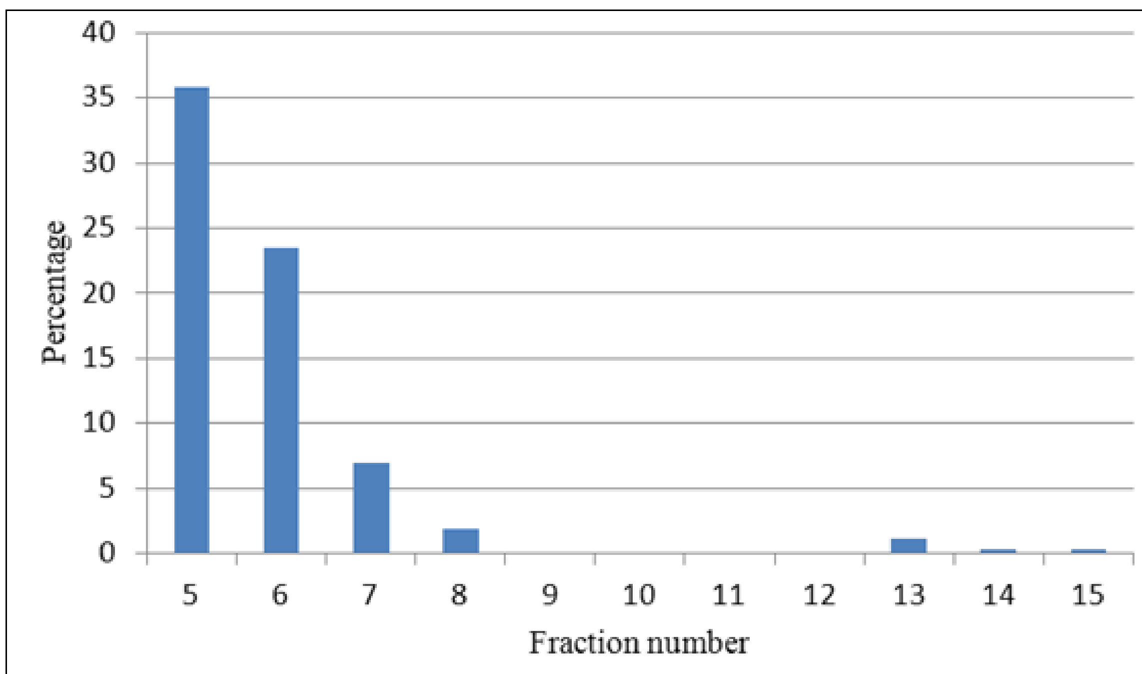


Figure 13: Determination of curcumin content in the fractions separated by size exclusion chromatography.

The amount of curcumin in samples was determined using UV-spectrophotometer at 425 nm.

4.4. Stability of Formulations

The accelerated stability test was preformed on empty liposomal formulation (control) and liposomal samples containing 10 mg of Curcuma extract, curcumin and curcumin I. The chosen formulations were prepared according to the section 3.2.1. and exposed to the same conditions e.g. sonication, centrifugation and temperature to insure comparative results as described in the section 3.2.8.

We could not observe the increase in mean particle size during the accelerated stability testing (Figure 14). On the contrary, in the case of some formulations, the actual size appeared to be smaller, which might be explained by the effect of temperature on the separation of agglomerated particles.

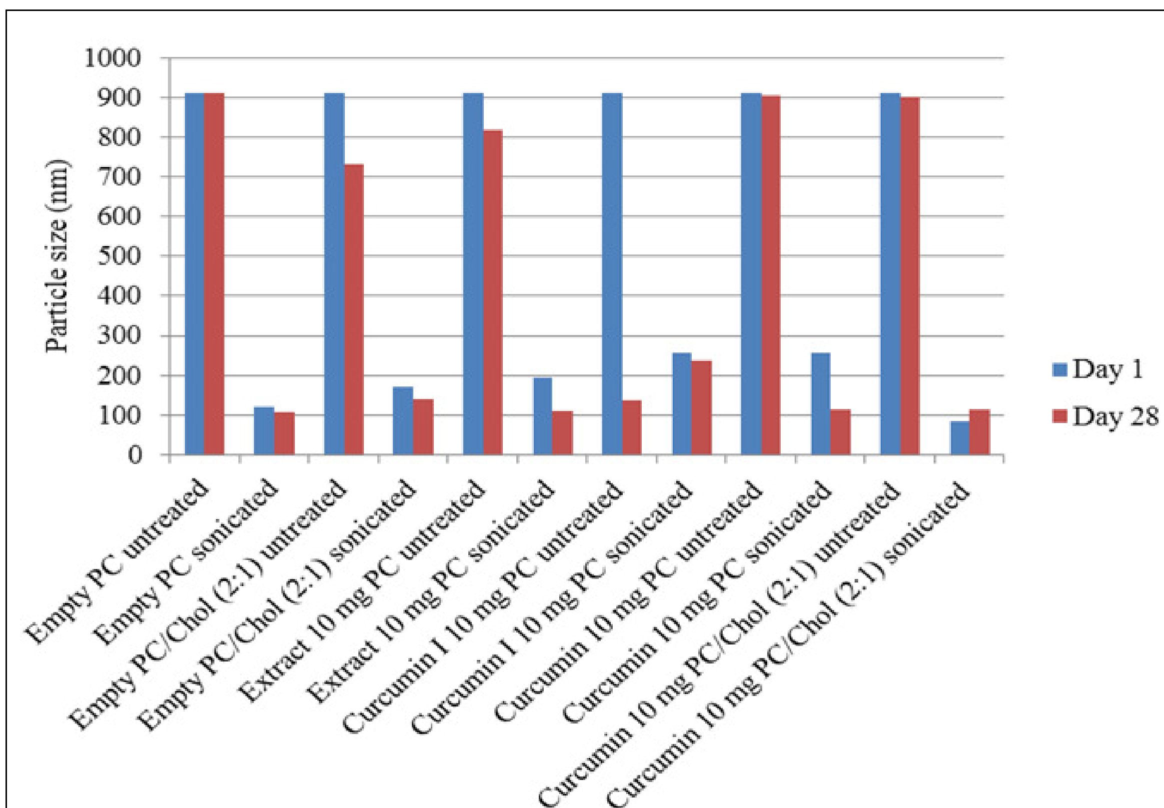


Figure 14: Changes in particle size during storage at 40 °C for 4 weeks.

Regarding the loss of entrapped curcumin during the accelerated stability testing, the findings in the stability experiment showed that the loss of drug was seen among the sonicated formulations. This finding is as we expected as unilamellar vesicles have tendency to lose the entrapped or exchange the entrapped material more easily than the multilamellar vesicles (New, 1990). The results presented in Figure 15 show significant loss of drug content for sonicated PC formulation with 10 mg curcumin. Apparently the incorporation of cholesterol increased the retention of curcumin in the liposomes and limited the changes in size seen with PC formulations, thus improved the stability. MLVs were clearly more stable than SUVs with respect to leakage.

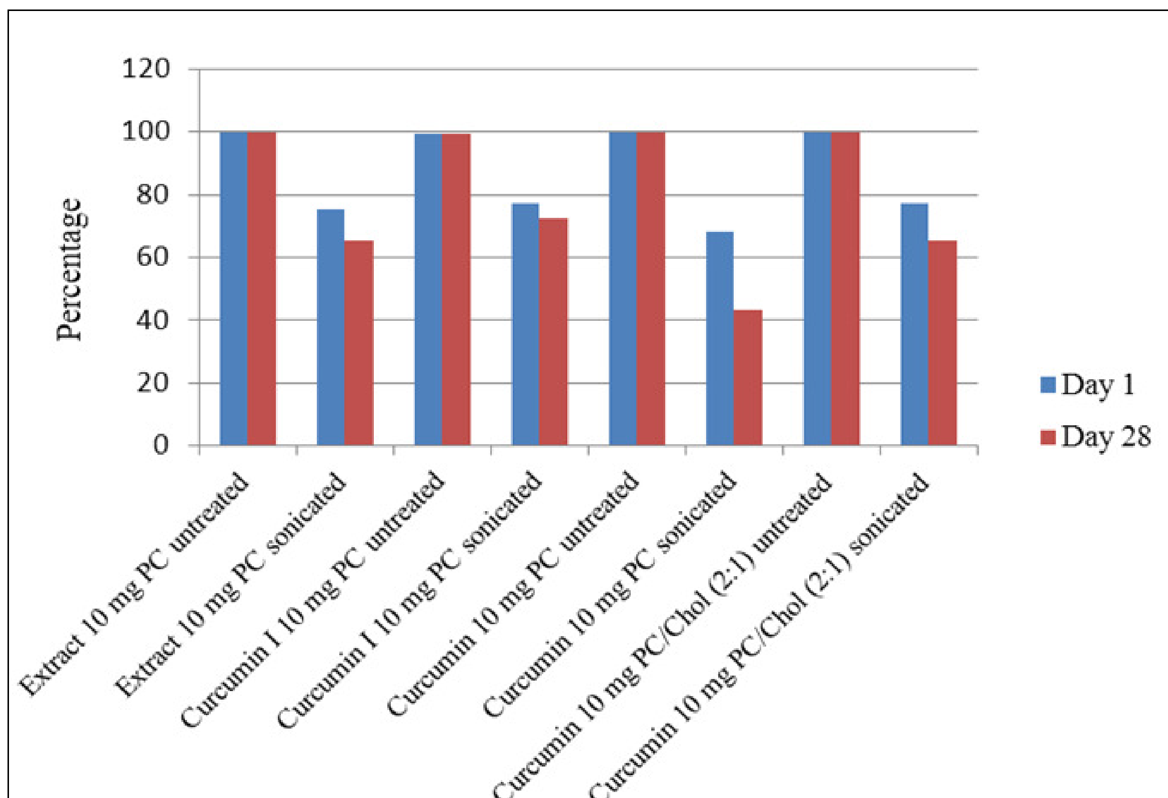


Figure 15: Loss of entrapped curcumin during the accelerated stability testing.

Visual examination of the samples showed that liposomes formed sedimentation, probably due to fusion and agglomeration of the liposomes. These agglomerations appeared to affect the recovery from the tested samples.

4.5. Antioxidant Activity of Curcumoids

The radical scavenging (antioxidant) activity was determined using the test described in section 3.2.9. The results presented in Figure 16 shows that liposomal formulations appear to have an antioxidant activity. Moreover, the mixtrure of curcumoids in curcmin appears to be more potent than the pure compound (curcumin I). This finding is suggesting that it is rather more cost-effective to use the mixture of curcumoids directly in the formulations than purifying the mixture to get pure compounds. However, this concept of using

mixtures could be difficult to apply in pharmaceutical industry, therefore it is necessary to standardize the composition of the mixture in order to obtain reproducible results.

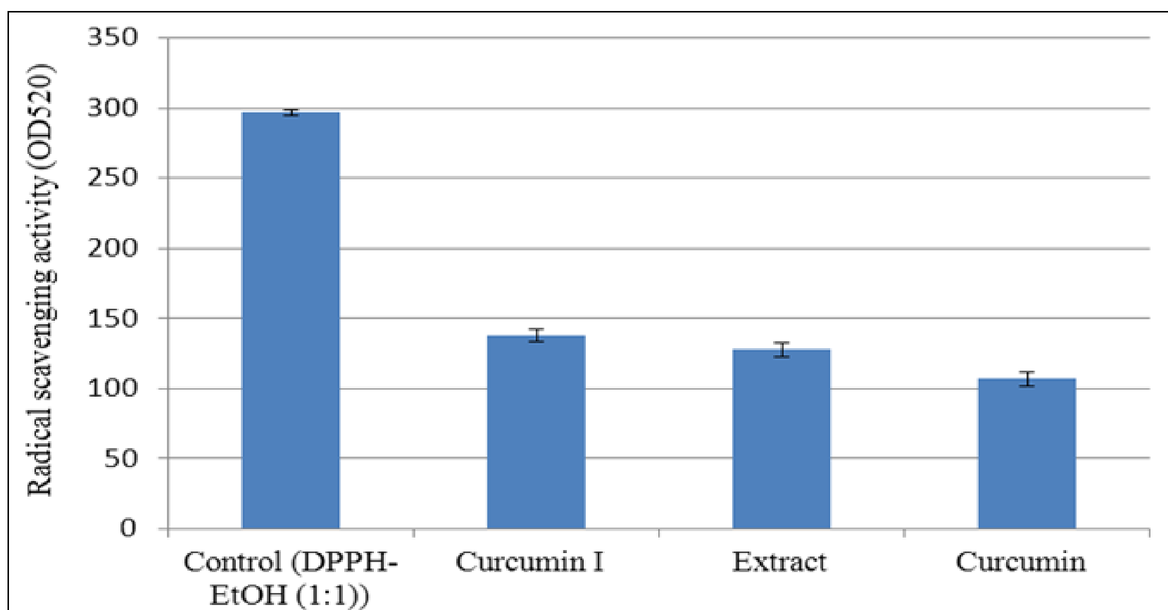


Figure 16: Radical scavenging activity of curcumoids.

A sample of 1 mL with 5 µg/mL of each liposomal formulation was mixed with 1 mL of methanolic solution of DPPH. The scavenging of DPPH radical was measured at 520 nm. Each value represents the mean of three readings.

5. CONCLUSION

We were able to entrap curcumin and curcumin from Curcuma extract successfully in liposomes. Moreover, the entrapment efficiency was found to be dependent on the ratio between phospholipids and curcumin used. The stability experiment showed that the characteristics of liposomes were dependent on liposomal compositions. The inclusion of cholesterol in liposomal membrane affected membrane rigidity as well as stability of vesicles. During the process, we discovered that the standard curcumin, commercially available, was not really pure compound, rather a mixture of three curcuminoids. However, DPPH radical scavenging activity study revealed that pure curcumin has less potency as compared to curcuminoids mixture and Curcuma extract. In regard to the development of industrial product, the liposomal formulations with curcumin or Curcuma extract would be more cost-effective than liposomal formulations with pure curcumin I.

6. FUTURE PERSPECTIVE

It would be of interest to optimize the stability of the formulations. Incorporation of liposomes into a vehicle with suitable viscosity and bioadhesiveness would modify the properties of liposomes and make the formulation more suitable for the topical application. In this case, adding liposomes into hydrogels such as Carpobol would be interesting since it could theoretically provide the desired properties. Moreover, it would be interesting to investigate the release profile of the drug from liposomes.

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8. APPENDIX

ABSTRACT SUBMITTED

LIPOSOMAL CURCUMIN: THE WAY TO IMPROVED ANTIINFLAMMATORY ACTIVITY

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Purpose: Curcumin (I), demethoxy curcumin (II) and bisdemethoxy curcumin (III) are commonly called curcuminoids and are major constituents of the widely used spice, turmeric powder (*Curcuma longa* rhizome). Curcumin has shown diverse pharmacological effects in treatment of various diseases due to its strong antioxidant and anti-inflammatory activities. Its therapeutic potential is acknowledged in over 10,000 research papers and over 40 clinical trials. Its broader use is limited by its poor solubility. We propose that drug delivery system such as liposomes can provide a mean of improved therapeutic action.

Methods: We standardized water-, alcoholic- and ethyl acetate-extracts of turmeric powder and characterized compounds I, II and III by HPLC, NMR and MS. The extracts with major curcuminoid constituents and/or individual compounds I, II and III were incorporated in liposomes. Formulations were optimized in regard to vesicle size, lipid composition entrapment efficiency and stability. The *in vitro* antioxidant and anti-inflammatory activities of free and liposomally entrapped curcuminoids were determined.

Results: Ethanol extract of turmeric powder contains almost exclusively three curcuminods. Liposomal preparation significantly enhanced *in vitro* anti-inflammatory activity, however antioxidant activity based on DPPH assay was not significantly changed. Ethanolic extract expresses stronger biological response than individual curcuminoids.

Conclusions: Use of turmeric powder extract in form of liposomal preparation is found to be superior and cost-effective. Anti-inflammatory activity of curcumin/curcuminoids enhanced by liposomal carrier makes the proposed system especially interesting for topical treatment of vaginal inflammation.