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***Research Article***


# Curcumin And Etoricoxib Encapsulated Liposomes: Formulation, Characterization And Anti-Inflammatory Effects In Rat Models.


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***Abstract***

To enhance anti-inflammatory efficacy of Curcumin (CURC) and Etoricoxib (ETRX); and to reduce their notorious side effects, they were loaded into liposomal formulations (LFs). The present study aimed at formulation, characterization and evaluation of anti-inflammatory effects of LFs of CURC in combination with ETRX in experimental acute inflammation (AI) in rat model induced by carrageenan administration. The existing pharmaceuticals for treating arthritis are analgesics, steroids and non-steroidal anti-inflammatory drugs (NSAIDs), which reduce the symptoms such as severe pain and inflammation. Classical NSAIDs are cyclooxygenase (COX) inhibitors that inhibit prostaglandins (PGs) and thromboxane synthesis, thereby reducing inflammation. New NSAIDs selectively inhibit COX-2 and are usually specific to inflamed tissue, which decreases the risk of peptic ulcer. However, their long-term use cannot be sustained due to inadequate pain relief, immune disturbances and serious gastrointestinal and cardiovascular adverse events. Therefore, plant-based product like CURC with anti-inflammatory properties and minimum side effects are needed for the treatment of arthritis, including rheumatoid arthritis (RA) and osteoarthritis, especially after the withdrawal of many Food and Drug Administration (FDA)-approved anti- inflammatory drugs. However, its poor solubility, low chemical stability and short half-life following systemic absorption

contribute to CURC being considered a pharmaceutical challenge. Numerous delivery systems have been proposed as means to tailor its biological properties. In this research, we are particularly interested in potential of CURC as an anti-inflammatory agent in combination with ETRX and delivery in the form of LFs. ETRX; a NSAID is proposed to treat inflammation in rat model as it is known for its anti-inflammatory, analgesic and antipyretic effects. ETRX, a widely prescribed anti- inflammatory drug belongs to class II under BCS (biopharmaceutical classification system) and exhibit variable oral bioavailability due to its poor aqueous solubility. This research is aimed to study synergistic effect of a natural compound CURC and an allopathic NSAID moiety ETRX to treat inflammation in rat model, by oral ingestion in the form of LF as an efficient drug delivery system. Hence, we propose LF as a mean to overcome the CURC limitations. Liposomes (LPs) encapsulation of CURC makes this formulation amenable to circumvent the problem of poor oral availability that limits the utility of free CURC. The LFs can potentiate the effects of encapsulated drugs by sustaining the release over an extended period of time. The results of our study demonstrated that the association of CURC with ETRX in the form of LFs could potentiate the anti-inflammatory effects in reduced doses *in vitro* and in rat model. The LFs were spherical in shape in TEM images at various resolutions. The particle size of optimized CURC-ETRX LPs was found to be 276.1 nm with PDI value of 24.5. The maximum EE for CURC and ETRX in optimized formulation (F2) was found 98.915% and 93.877% respectively. The % EE of CURC-ETRX loaded LFs was found to be dependent on the lipid concentration, resulting almost quantitatively for a maximum 15 mg of total lipids (PC+CL) and progressively decreasing at higher 20 mg of total lipid (PC+CL) content, may be due to precipitation of drugs at higher lipid concentrations. The cumulative percentage release of CURC and ETRX from optimized formulations was found to be 59.64% and 83.11% respectively, for a period of 24 hours. We investigated the *in vivo* effect of CURC and ETRX loaded LF on local edema in carrageenan-induced paw edema in rat model. The percentage inhibition ofedema in rat model was found to be better for CURC-ETRX LF in comparison to conventional CURC and ETRX in solution forms ($p<0.05$). Hence, the association of CURC and ETRX to a low dose in the form of LFs could be an appropriate combination to decrease NSAID doses used to reduce pain, inflammatory cytokines, and histological changes in AI.



## INTRODUCTION

This study is aimed to investigate the formulation, characterization and evaluation of anti-inflammatory effect of liposomal formulation (LF) of Curcumin (CURC) and Etoricoxib (ETRX) as oral drug-delivery system. In this study, the combination of CURC and ETRX in the form of LFs is proposed as an efficient alternative to treat inflammation.

Arthritis is a form of joint disorder characterized by chronic inflammation in one or more joints that usually results in pain and is often disabling. Inflammation is a broad and ancient medical term referring to a set of classic signs and symptoms, including pain, edema, warmth and loss of function [1,2]. It is characterized by a group of complex changing responses to tissue injury primarily caused by harmful stimuli, such as pathogens, physical agents, chemical compounds or damaged cells.

Carrageenan is a pro-inflammatory polysaccharide used to induce local inflammation (paw edema). The release of bradykinin, serotonin, histamine and prostaglandins (PGs)under cyclooxygenase enzymes (COX) occurs in the early phase of inflammation. In the late phase, PG generation is continued together with neutrophil infiltration and release of pro-inflammatory cytokines such as tumor necrosis factor (TNF-α) and interleukin-1 (IL-1β). The overproduction of neutrophil-derived free radicals and nitricoxide is also involved in the delayed phase of carrageenan-induced acute inflammation(AI) [3]. It was suggested that drugs targeting the COX enzyme, pro-inflammatory expression (e.g., inducible nitric oxide synthase; iNOS) and free radical formation might better control the inflammation process.

Nonsteroidal anti-inflammatory drugs (NSAIDs) are one of the most commonly prescribed medications for acute and chronic inflammation. Their major therapeutic actions are related to their ability to block certain PGs

synthesis through COX-1 and COX-2 enzymes inhibition. COX-1 is expressed in normal cells and produces PGs andthromboxane A2, which control renal homeostasis, platelet aggregation and mucosal barrier in the gastrointestinal tract and possess other physiological functions. COX-2 isinduced in inflammatory cells and produces PGs related to inflammation, pain, and fever [4,5]. The inhibition of COX-2 most likely represents the desired effect of NSAIDs by providing anti-inflammatory, analgesic andantipyretic responses; inhibition of the COX-1 enzyme plays a major role in the undesired side effects such as injury to the gastrointestinal mucosa. Therefore, in some circumstances, NSAID administration may cause acute renal failure, gastrointestinal ulcers, hypertension, or serious cardiovascular events (such as stroke or acute myocardial infarction). These adverse effects may be prevented first bylimiting NSAID dosage and duration of administration and also by performing risk assessments for each patient depending on the associated pathology. Hence, NSAIDs should be replaced by natural compounds like CURC. Hence, this research project is aimed to study synergistic effect of a natural compound CURC and a NSAID ETRX totreat inflammation in rat model, by oral ingestion in the form of LFs as drug delivery system**.**

ETRX, a NSAID is proposed to treat inflammation in rat model as it is known for its anti-inflammatory, analgesic and antipyretic effects. ETRX, a widely prescribed anti- inflammatory drug belongs to class II under BCS and exhibit variable oral bioavailability due to its poor aqueous solubility [6].

CURC, primary component of the spice turmeric extracted from the rhizomes of *Curcuma longa*, represents the major anti-oxidant and anti-inflammatory substance found in turmeric, acting through various mechanisms; and is pharmacologically safe. In fact, clinical trials showed that the CURC can be administered to the patients up to 8,000 mg/day without adverse effects. The beneficial effects of CURC are limited dueto its hydrophobic characteristics, as CURC is not soluble in water; is poorly absorbedin the small intestine and it has an extensive reductive and conjugative metabolism in the liver. CURC is widely prescribed as anti-inflammatory drugs, but reported with poor bioavailability and low solubility. Hence, there is need of alternative oral formulation of CURC, which can improve its oral bioavailability issue.

Transdermal drug delivery has been proposed as a suitable administration routefor CURC [7]. However, the highly hydrophobic properties and the excellent barrier function of the skin lead to a very low percutaneous penetration of CURC, which makes developing a transdermal-delivery system for CURC a challenge [1]. From literature review, topical LPs (liposomes) of CURC are reported but there is very less information about oral LFs. Also, the combination of CURC and ETRX is not reported. To provide potential delivery of these drugs, LPs are proposed as an efficient delivery system. The LPs are small artificial vesicles of spherical shape with a membrane composed of phospholipids bilayers [8]. .The main component of LPsare phospholipids and CL stabilized by emulsifiers, the kind of phospholipids can directly influence the physical properties of LPs. They are widely used as carriers, especially in their application to topical and oral delivery for a variety of drugs, becauseof their small size, biodegradability, hydrophobic and hydrophilic character and low toxicity [9,10]. Results from several studies demonstrate thatLPs have the potential to enhance drug absorption, improve therapeutic effectiveness, reduce serious side effects, and for the sustained release of drug.

In order to take advantage of these properties, our study aimed to develop CURC and ETRX loaded LPs, which could enhance the absorption, can sustain the release over anextended period of time with less side-effects. These LPs were prepared by thin film hydration method followed by probe sonication. Phosphatidycholine (PC), pluronic (PL) and CL (Cholesterol) were selected for the preparation of CURC and ETRX loaded LPs. LFs were made of PC and PL (total 100 mg) in a weight ratio of 1:1, 2:1 and 3:1 respectively. The ratio of PL and CL were fixed at 1:1 w/w in all theformulations. The formulations were optimized on the basis of particle size and Entrapment Efficiency (EE). The *in vitro* drug release studies were carried out with Franz diffusion cell using cellulose membrane. Various mathematical models were applied to find out release patterns and best fit. The stability studies were carried out atrefrigerator temperature of 5±3°C and at ambient temperature of 25±2°C at 60 ±5% relative humidity (RH) in a stability chamber for two months, to find out any physical changes on storage.

The present study evaluated the anti-inflammatory effects of CURC-ETRX LFs in comparison to conventional CURC and ETRX in experimental carrageenan induced paw edema in rat model. The optimized formulation displayed the greatest ability to reduce the inflammation in rat models in comparison to conventional formulation. The results of our study demonstrated that the association of CURC with ETRX in theform of LF could potentiate the anti-inflammatory effects in reduced doses *in vitro* andin rat model. The LFs obtained are of nanometer size and spherical in shape in TEM images at various resolutions. The nanometer size range and low PDI values were obtained for LPs. The particle size of optimized CURC-ETRX LPs was found to be 276.1 nm with PDI value of 24.5. The maximum EE for CURC in optimized formulation (F2) was found 98.915% and 93.877% respectively. The high EE is due tohigh affinity of lipophilic drugs toward the lipoidal matrix of PC and CL with maximumstabilization provided by the surfactant to the lipid core. The cumulative percentage

release of CURC and ETRX from optimized formulations was found to be 59.64% and 83.11% respectively, for a period of 24 hours. The LF preparation has shown sustainedrelease profiles over an extended period of time *in vitro* and *in vivo* models which in turn resulted in better therapeutic outcome. The results of animal study demonstrated that the association of CURC with ETRX reduced the inflammation level in reduced doses in the form of LFs; because CURC can enhance the anti-inflammatory effect of ETRX through inhibition of PG synthesis via the COX pathway.

We investigated the *in vivo* effect of CURC and ETRX loaded LF on local edema in carrageenan-induced paw edema in rat model. Four groups were investigated: normal control, disease control, standard control and test control. The percentage inhibition ofedema in rat model was found to be better for CURC-ETRX LF in comparison to conventional CURC and ETRX in solution forms ($p<0.05$). The rats treated with CURC-ETRX LF showed highest 85.36 percentage inhibition up to 6 hours, in comparison to conventional CURC and ETRX providing 72.27 level of inhibition, up to4 hours only. Hence, the synergistic effect of CURC and ETRX in the form of nanosizedLF was successful as better anti-inflammatory formulation to reduce paw edema at lowdose in rat models; also, the release profile was sustained up to 6 hours in comparison to 4 hours release profile achieved with conventional formulations. CURC nanoparticles were already demonstrated to provide superior anti-inflammatory effectsin an animal model of acute and chronic inflammations when given orally. It is concluded that by reducing CURC particle size to the nanoscale in the form of LFs, thetherapeutic efficacy is increased. The optimized LFs were found to be stable on testingat refrigerator temperature of 5±3°C and at ambient temperature 25±2°C at 60 ±5% RHin a stability chamber for a period of 3 months.

The present study evaluated the anti-inflammatory effects of LFs of CURC in combination with ETRX in experimental AI induced by carrageenan administration. Inthis study, the combination of CURC and ETRX in the form of LF is proposed as an efficient alternative in comparison to conventional CURC and ETRX formulations. Hence, the association of CURC and ETRX to a low dose in the form of LF could be an appropriate combination to decrease NSAID doses used to reduce pain, inflammatory cytokines and histological changes in AI.

## 1. METHODOLOGY

In this research, the method adopted for preparation of drug loaded LPs is 'thin film hydration method' using PC as lipid and PL as surfactant.

### 1.1. PREFORMULATION STUDIES

The drug samples of CURC and ETRX were identified by various analytical techniques:

**i. Standard curve and UV Spectroscopy**

CURC was accurately weighted and transferred into the volumetric flask. The volumetric flask was filled up to the mark with pH 7.4 phosphate buffer and ethanol (1:1) mixture. The clear solution was obtained by sonication of the solution on bath sonicator. The standard stock solution of ETRX was prepared by dissolving 0.1N HCl to make final concentration of 100 µg/ml. CURC and ETRX solution was scanned in UV spectrophotometer within the wavelength range of 200-800 nm and 200-400 nm respectively. The λmax was found to be 429 nm for CURC and 233 nm for ETRX [11,12].

ii.**Solubility studies**

The solubility of CURC and ETRX in water, physiological buffer and various organic solvents were determined by adding an excess quantity of drug in the solvents [13,14]. Using a water bath shaker (Swastika, India), the suspensions were stirred for 48 hrs at 37°C, filtered and the samples were analysed using Shimadzu Corporation, LC2010C HT spectrophotometer for analysis of drug content after suitable dilution. All samples were analysed in triplicates.

**iii.Melting point**

The melting point determination was done by capillary method. A small amount of drug was placed in capillary tube [15]. The temperature at which melting started and the temperature at which complete melting occurred, were noted.

### 1.2. DRUGS- EXCIPIENTS COMPATIBILITY STUDIES

**i.Physical Mixing**

Compatibility studies between drugs and PC were carried out by physical mixing. The mixing was evaluated for any physical change on mixing.

**ii.FTIR studies**

The FTIR (Fourier Transformer Infrared) spectra of pure drugs and physical mixtures were compared to check

any interaction between drugs and selected lipid, over a spectral region from 4000 to 400 $cm^{-1}$, using PERKIN-ELMER FTIR spectrophotometer.

### 1.3. METHOD OF PREPARATION OF DRUG LOADED LPs

#### 1.3.1 Materials

CURC (purity > 95%) was purchased from Saptamveda Pvt. Ltd. ETRX was provided as gift sample from Dr Reddy Laboratories; Ahmedabad. PC was purchased from Chempure Pvt, Ltd (Bangalore, India). PL F-127 was supplied by Otto chemie Pvt. Ltd (Mumbai, India).
CL was purchased from National Chemical Vadodara, (Gujrat, India). All other chemicals and solvents were of analytical grades.

#### 1.3.2 PREPARATION OF LPs

In this study, CURC and ETRX loaded LFs were prepared by conventional thin film hydration method followed by sonication [16, 17, 18].

### 2.1 Optimization of formulations

In this study, the selected range of lipid concentration was 5 -15 mg (w/w) lipid per 100mg of total formulation weight and the selected emulsifier concentration was 5 mg (w/w) per 100mg of total formulation. LFs were made of PC, PL and CL (total 100 mg) in a weight ratio of 1:1, 2.1 and 3:1 respectively. The ratio of PL and CL were fixed at 1:1 w/w in all the formulations. The optimization of best formulation was done on the basis of EE and particle sizes. On the basis of EE, the formulations F2 and F3 were selected. Then the particle sizes of these two formulations were determined using Malvern Zetasizer. On the basis of particle sizes, the F2 formulation with narrow size was selected as best formulation as it showed maximum EE and small particle size. A complete overview of the liposomal composition is given in Table 1; and the results of EE and particle size are summarized in Table 5 and 6.
The encapsulation efficiency (EE) of CURC-ETRX loaded LFs was found to be dependent on the lipid concentration, resulting almost quantitatively for a maximum 15 mg of total lipids (PC+CL) and progressively decreasing at higher 20 mg of total lipid (PC+CL) content, may be due to precipitation of drugs at higher lipid concentrations.

**Table 1:** Composition of various formulations

| Formulation | CURC | ETRX | PC | PL | CL | %EE(CURC) | %EE(ETRX) |
|---|---|---|---|---|---|---|---|
| **F1** | **50** | **25** | **5** | **5** | **5** | 97.315±0.009 | 91.577±0.072 |
| **F2** | **50** | **25** | **10** | **5** | **5** | 98.915±0.009 | 93.877±0.072 |
| **F3** | **50** | **25** | **15** | **5** | **5** | 98.715±0.006 | 92.377±0.072 |

**Table 2:** Particle size and PDI values of F2 and F3

| Formulation | Particle size | PDI value |
|---|---|---|
| F2 | 276.1 nm | 24.5 |
| F3 | 326.7 nm | 25.1 |

### 2.2 Formulation of LPs

The steps in formulation of LPs by conventional thin film hydration method followed by sonication are summarized as follow: [16,17,18].

- The calculated amounts of CURC and ETRX were dissolved in methanol containing CL in round bottom flask of Rota evaporator (EQUITRON ROTEVA, MEDICA INSTRUMENT). The solvent was evaporated under reduced pressure at temperature $70^0$C using a rotary evaporator resulting in the formation of a thin film.
- To ensure the complete removal of the residual solvent, the flask was left overnight in vacuum desiccators.
- The thin lipid film was hydrated by addition of citrate buffer of pH 3.4 at room temperature. Subsequently, the obtained suspension was shaken properly in the flask and incubated for 2-3 hours at room temperature for complete hydration of the lipid film.

- The liposomal suspension was kept in the refrigerator to mature overnight at $4^0$C.
- The LFs were sonicated using (model 750F, PCI Analytics Pvt. Ltd., Mumbai) for 1 min. Probe tip sonicators transfer high energy to the lipid dispersion, which can result in reduction in size of formulation to nanoscale range.
- Using this approach, the sonicator tip is placed in a sample tube containing the dispersion and the sonication process is typically set to 3 s with a pause of 7 s between two ultrasound bursts.
- The mixture was sonicated with probe sonicator with an ultrasonic probe at 20% ultrasonic power amplitude for 30 seconds to form LFs.
- The end product was uniform LF with smooth consistency.

## 2.3CHARACTERIZATION OF LPs

### 2.3.1 Transmission electron microscope (TEM)

The optimized formulation was characterized for its shape by transmission electron microscopy using a 300 mesh carbon-coated copper grid and phosphotungstic acid (1%; w/v) as a negative stain [18]. After being stained, the samples were allowed to dry at room temperature for 10 min for investigation.

### 2.3.2 Particle size determinations

Particle size and polydispersity index of dispersion were measured by a dynamic light scattering process using zetasizer (Malvern zetasizer). Vesicle properties, particle size diameter were determined at room temperature. For the particle size measurements, the dispersion was suitably diluted with distilled water in order to avoid multi scattering phenomena [19,20].

### 2.3.3 Determination of Entrapment Efficiency (EE)

The percentage drug entrapped in the CURC and ETRX loaded LF was determined by the centrifugation at 5000 rpm for 15 min using a centrifuge (REMI R8-C,REMI MOTORS, MUMBAI) to separate the loaded drug from free drug [17,21]. Then, supernatant was separated and analysed after suitable dilution in solvent by UV-Visible spectrophotometer at 425nm and 233nm which indicates the amount of free drug. The LPs (sediment) was redispersed in same solvent (methanol) and analysed drug content after dilution using UV.

### *2.3.4 In vitro* release studies for drug loaded formulations

#### 2.3.4.1 Diffusion cell method

The *in vitro* drug release studies were carried out with Franz diffusion cell using cellulose membrane [22, 23]. Franz diffusion cell (model 2351-6C, KSHITIJ INNOVATIONS, HARYANA, INDIA) was used for *in vitro* drug release studies. The cellulose membrane separating the donor compartment from the receptor compartment was placed in between the donor and receiver cells. The receptor compartment contained phosphate buffer solution of pH 7.4 for analysis of permeated drugs. About 1 ml of LF was taken in the donor compartment (Figure 1).The samples were removed using the micro syringe at specific intervals of time upto 24 hours as shown in Figure 1. To keep the sink condition, every time the sample withdrawn was replaced with same quantity of fresh buffer maintained at same conditions [24,25]. The amount of drug released *in vitro* was estimated using UV spectrophotometer at 429nm and 233nm for CURC and ETRX respectively.

**Figure 1: Image of Franz Diffusion Cell**

#### 2.3.4.2 Release kinetic models for best fit of release

To find out the patterns and best fit for release of drugs from LF, various mathematical models were applied. These models for data fitting were zero order release model, first order release model, Higuchi model and

Korsmeyer-Peppas models. The best fit was decided on the basis of highest correlation factor.

### 2.3.5 STABILITY STUDIES

The optimized LFs were subjected to stability testing at refrigerator temperature of 5±3°C and at ambient temperature 25±2°C at 60 ±5% RH in a stability chamber (Tidar, Model 80X80X25) for a period of 3 months [26]. The parameters used to access the stability of LF were: variations in appearance, consistency, phase separation and pH of formulation.

## 2.4ANTI-INFLAMMATORY AND ANALGESIC EFFECTS OF CONVENTIONAL AND LF OF CURC AND ETRX USING CARRAGEENAN INDUCED PAW EDEMA IN RAT MODEL

The present study evaluated the anti-inflammatory and analgesic effects of conventional CURC and ETRX in experimental AI induced by carrageenan administration. Four groups of six randomly selected Wistar rats was evaluated. The optimized formulation displayed the greatest ability to reduce the inflammation in rat models in comparison to conventional formulation.

### 2.4.1 Materials

Carrageenan and carboxylmethyl cellulose (CMC) were purchased from S.D. Fine- Chem. Ltd, Mumbai. Normal saline was purchased from local supplier.

### 2.4.2 Animals

All animals were approved by Institutional Animal Ethical Committee (IAEC). Adult male and female Wistar rats (200-250grams) were used for the studies. The animals were housed in standard polypropylene cages (two animals/cage) and maintained under controlled room temperature (22 ±1°C) with a 12:12h light and dark cycle. Water and food were available *ad libitum*.

### 2.4.3 Animal groups and treatments

The experimental protocol was approved by the Institutional Animal Ethical Committee (IAEC) and conducted according to the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA). GHG/2023/IAEC/P02/M06 is the project proposal number. A single dose *in vivo* study was designed and adult Wistar rats (200-250 grams) were used for the study. The animals were divided into four groups (n=4) as shown in Table 3.

Group 1: Normal Control Group 2: Disease Control Group 3: Standard Control Group 4: Test Control

**Table 3:** Different groups and treatment given to the animals

| S.no. | Group | Treatment | Species | No. of animal required |
|---|---|---|---|---|
| 1. | Normal control | No treatment will be given | Wistar rats | 6 |
| 2. | Disease control | Carrageenan | Wistar rats | 6 |
| 3. | Standard control | Carrageenan + CURC(50mg/kg) and ETRX (25mg/kg) solution | Wistar rats | 6 |
| 4. | Test Control | Carrageenan + CURC(50mg/kg) and ETRX (25mg/kg) LF | Wistar rats | 6 |

### 2.4.4 Carrageenan induced Paw edema model

#### 2.4.4.1. Preparation of 1% carrageenan suspension

1% suspension of carrageenan was prepared by sprinkling 100 mg of carrageenan powder in 10 ml of saline (0.9 % w/v NaCI). Solution was set aside and soaked for 1 hour. A homogenous suspension was then obtained by thorough mixing with magnetic stirrer.

#### 2.4.4.2. Method of Percentage inhibition of Paw edema

- Anti-inflammatory response was measured by rat paw edema, it is based on principle of release of various inflammatory mediators by carrageenan [3,27].
- A mark was made on right hind paw just below the tibia-tarsal junction so that every time the paw dipped into the column of the plethysmometer up to the mark to ensure a constant paw volume.
- After 60 min of the above treatment an inflammatory edema was introduced in the right hind paw by injecting 0.1ml carrageenan (1% in the planter tissue of the paw of all animals).
- The initial paw served as a reference to non-inflamed paw for comparison. The initial paw volume was

measured with plethysmometer within 30 sec of the injection, the relative increase in the paw volume was measured in normalcontrol, disease control, standard and treated groups at different time interval 0 min, 1hour, 2 hour, 3 hour, 4 hour, 5 hour and 6 hour after carrageenan injection.

- The inflammation in paw volume is calculated, compared with the basal volume interval and calculated as percentage compared with the basal volume.
- The difference of average values between treated animals and control group is calculated for each time and evaluated statistically.
- The percentage inhibition of paw edema was calculated by using the following formulas:

Percentage of edema Inhibition = (Vc-Vt/Vc) x 100 Vc- Volume of paw edema in control group
Vt-Volume of paw edema in treated group

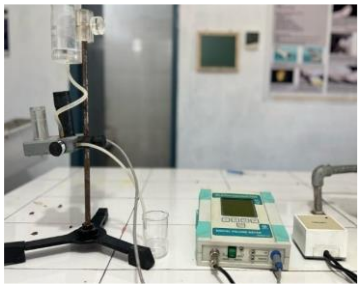

Figure2: Image of Digitalplethysmometer

Figure 3: Image of measurement of paw edema using digital plethysmometer

## 3. RESULTS AND DISCUSSION

### 3.1. PREFORMULATION STUDIES

#### 3.1.1. Standard Curve and UV spectroscopy

The value of λmax of drugs were determined in phosphate buffer 7.4 and 0.1N HCL by scanning the sample solution in the range 200 nm to 800nm at 1 cm path length using UV/visible spectrophotometer (Shimadzu Corporation, LC2010C HT). CURC and ETRX showed maximum absorption (λmax) at 429 nm and 233 nm respectively. The standard plot in phosphate buffer pH 7.4 and ethanol (1:1) of CURC and in 0.1N HCL of ETRX were prepared.

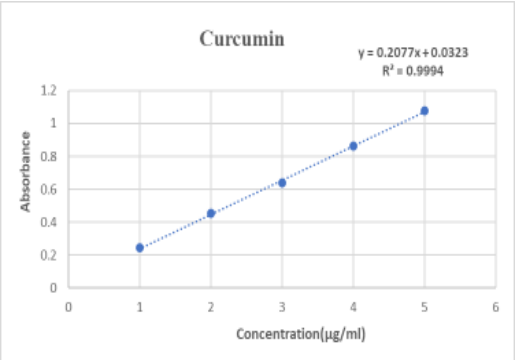


Figure4: Standard Calibrationcurve of CURC in 0.1N HCL

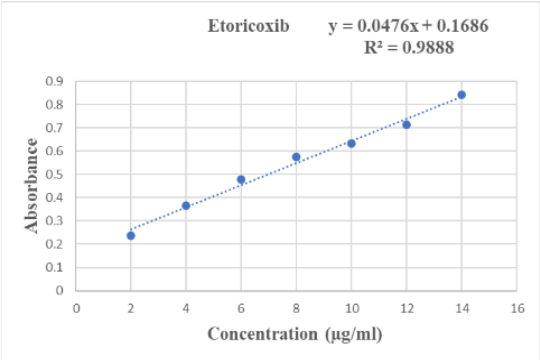


Figure 5: Standard Calibration curve of ETRX in 0.1N HCL

#### 3.1.2. Solubility Studies

The solubility studies of drugs were carried out in water, physiological buffers and methanol at 37°C under continuous stirring for 24 hours with the help of water-bath shaker (Swatika, India). The aliquots were withdrawn and analysed for drug content by UV spectroscopy. CURC is soluble in methanol and phosphate buffer 7.4 whereas insoluble in water. The results are shown in Table: 4. ETRX is freely soluble in methanol and insoluble in water and phosphate buffer.

**Table 4:** Solubility of CURC and ETRX in various solvents

| Sr.no. | Solvents | CURC (mg/ml) | ETRX(µg/ml) |
|---|---|---|---|
| 1 | Water | 0.12 | 24.35 |
| 2 | Methanol | 10.01 | 84.88 |

| | | | |
|---|---|---|---|
| 3 | Phosphate buffer 7.4 | 3.5 | 79.23 |

## 3.2. DRUG-EXCIPIENTS COMPATIBILITY STUDIES

### 3.2.1. Physical Mixing

In physical mixing, no physical changes were observed on mixing of drugs and excipients at low and high ratio. Hence, no physical interactions were seen.

### 3.2.2. FTIR Studies

FTIR Analysis of drug and excipient mixture was done using FTIR spectrophotometer (PERKIN-BLMER). No significant changes were observed in FTIR spectra, confirming no chemical interactions.

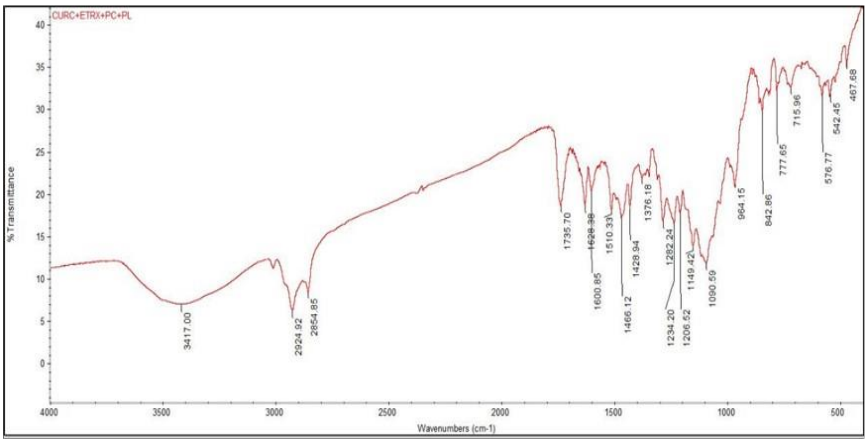


**Figure 6: FTIR spectrum of combination of CURC+ETRX+ PC+PL**

## 3.3. PREPARATION OF LPs

CURC and ETRX loaded LPs were prepared by thin film hydration method followed by probe sonication. PC, PL and CL were selected for the preparation of CURC and ETRX loaded LPs. LFs were made of PC and PL (total 100 mg) in a weight ratio of 1:1, 2:1 and 3:1 respectively. The ratio of PL and CL were fixed at 1:1 w/w in all the formulations.

The optimization of best formulation was done on the basis of EE and particle sizes. The results are summarized in Table 5 and Table 6. The EE which is ratio of the drug encapsulated to that of total drug loaded was calculated for the prepared three LFs. The maximum EE of 98.915% and 93.877% (f2) was observed for formulation F2 as shown in Table 6. On the basis of EE, the formulations F2 and F3 were selected. Then the particle sizes of these two formulations were determined using Malvern Zetasizer. On the basis of particle sizes, the F2 formulation with narrow size was selected as best formulation as it showed maximum EE and small particle size.

## 3.4. CHARACTERIZATION OF LPs

### 3.4.1. Transmission electron microscope (TEM)

**3.4.2.** The LPs prepared are of small size and spherical in shape in TEM images at various resolutions.

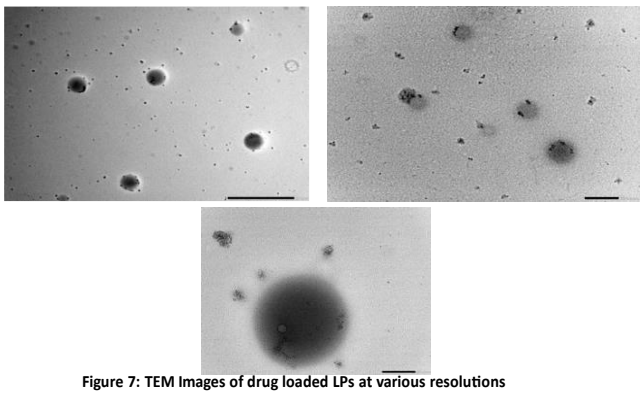

**Figure 7: TEM Images of drug loaded LPs at various resolutions**

### 3.4.3. Particle Size determinations

The nanometer size range and low PDI values were obtained for LPs. The particle size of optimized CURC-ETRX LPs was found to be 276.1 nm with PDl value of 24.5. The results of particle size of optimized LFs along with PDI values are summarized in Table 5. Particle size, PDI and Zeta Potential for CURX-ETRX LFs results are as shown in Figure 8, 9.

**Table 5:** Particle size and PDI values of F2 and F3

| Formulation | Particle size | PDI value |
|---|---|---|
| F2 | 276.1 nm | 24.5 |
| F3 | 326.7 nm | 25.1 |

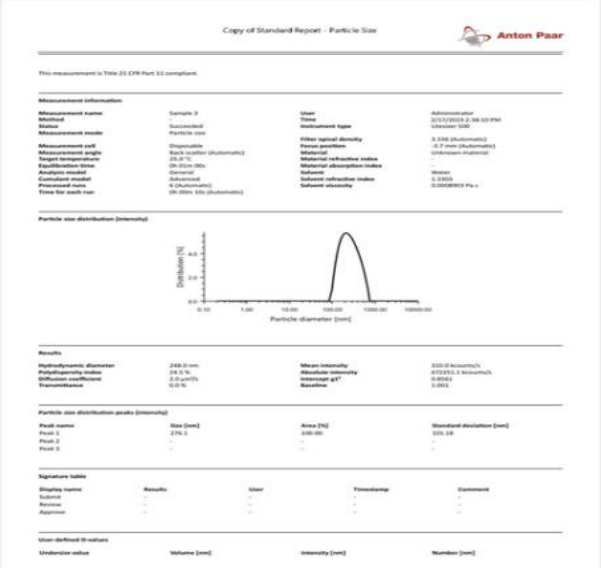

**Figure 8: Results of particle size and PDI of F2**

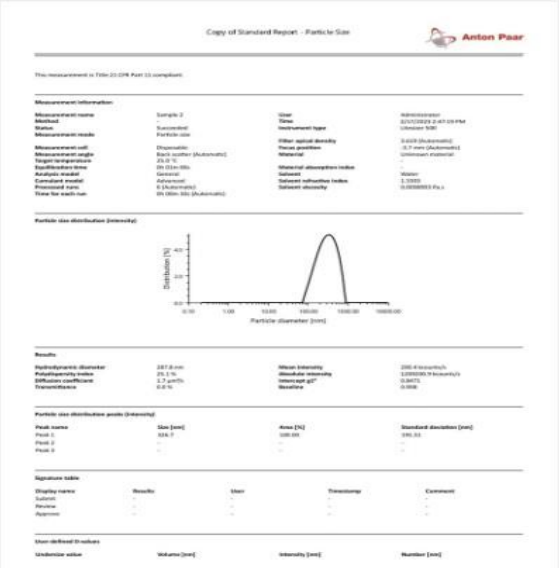

**Figure 9: Results of particle size and PDI of F3**

### 3.4.4. Determination of Entrapment Efficiency (EE)

The EE which is ratio of the drug encapsulated to that of total drug loaded was calculated for the prepared three LFs. The maximum EE of 98.915% and 93.877% (f2) was observed for formulation F2 as shown in Table 6. The %EE of CURC-ETRX loaded LFs was found to be dependent on the lipid concentration, resulting almost quantitatively for a maximum 15 mg of total lipids (PC+CL) and progressively decreasing at higher 20 mg of total lipid (PC+CL) content, may be due to precipitation of drugs at higher lipid concentrations. The high EE is due to high affinity of lipophilic drugs toward the lipoidal matrix of PC and CL with maximum stabilization provided by the surfactant to the lipid core.

**Table 6: %** Entrapment efficiency (EE) of LFs

| Sr. No. | Formulation Code | %EE |
|---|---|---|
| **1** | Sample 3 (CURC)(F2) | **98.915±0.009** |
| **2** | Sample 3 (ETRX)(F2) | **93.877±0.072** |
| **3** | Sample 2 (CURC)(F3) | **98.715±0.006** |
| 4 | Sample 2 (ETRX)(F3) | **92.377±0.072** |

### 3.4.5. *In vitro* release studies of drug loaded LFs

#### 3.4.5.1. Diffusion cell method

The *in vitro* drug release studies for the optimized formulation were carried out using cellulose membrane and diffusion cell apparatus. The cumulative percentage release of drugs was calculated and plotted versus time as shown in Figure 10. The cumulative percentage release for CURC and ETRX from optimized formulation was found to be 59.64% and 83.11% respectively, for a period of 24 hours. The results of *in vitro* release studies confirmed sustained release of CURC and ETRX over a period of 24 hours. Hence, LPs of CURC and ETRX can efficiently sustain the effects of these drugs for a long period of time and can provide better therapeutic outcome.

#### 3.4.5.2. Release kinetic models for best fit of release

The results of mathematical models for data fitting for release of drugs from the LFs, were calculated by applying zero order, first order, Higuchi model and Korsmeyer- Peppas model. The highest value of regression coefficient ($R^2$- 0.9293) was observed for zero order (Table 7) and hence, the best fit for release profile of CURC-ETRX LF is explained in terms of Zero order release model. The highest values was found for CURC and ETRX zero order model was applied.

**Table 7**: Curve fitting data of release rate profile for optimized formulation

| Mathematical Models | R2 | |
|---|---|---|
| | CURC | ETRX |
| Zero order | 0.9208 | 0.9232 |
| First order | 0.9121 | 0.8735 |
| Higuchi model | 0.7686 | 0.7638 |
| Korsemeyer Peppas model | 0.880 | 0.8686 |

### 3.4.6. Stability studies

The optimized LFs was found to stable at refrigerator temperature of 5±3°C and at ambient temperature 25±2°C/ at 60 ±5% RH in a stability chamber (Tidar, Model 80X80X25) for a period of 2 months. There was no significant change in the colour and consistency of the formulation during the course of stability studies but the pH of the formulation showed slight change from 6.1 to 6.3. Hence, the optimized LFs were found stable and no aggregation and precipitation was observed w.r.t time.

## 3.5 ANTI-INFLAMMATORY AND ANALGESIC EFFECTS OF CONVENTIONAL AND LF OF CURC AND ETRX USING CARRAGEENAN INDUCED PAW EDEMA IN RAT MODEL

All rats presented a marked unilateral peripheral paw edema after carrageenan administration. The paw volume presented a progressive increase, reaching the maximum values at 6 hours' time point, with the highest values in the rats from DC group. The rats were treated with the standard drugs ETRX, CURC and CURC-ETRX LF at dose of 50mg/kg and 25mg/kg for CURC and ETRX respectively. From findings, it was observed that the formulations showed significant decrease in inflammation in the carrageenan-induced paw edema rats. The rats treated with CURC-ETRX LF showed highest 85.36 percentage inhibition up to 6 hours, in comparison to conventional CURC and ETRX providing 72.27 level of inhibition, up to 4 hours only as shown in Figure 11. Hence, the synergistic effect of CURC and ETRX in the form of nanosized LF was successful as better anti-inflammatory formulation to reduce paw edema at low dose in rat models. The percentage inhibition of edema in rat model was found to be better for CURC-ETRX LF in comparison to conventional CURC and ETRX in solution forms ($p < 0.05$).

| S. No. | Group (n=4) | Paw edema volume(1hr s) Mean + SEM (% edema inhibition) | Paw edema volume(2hr s) Mean + SEM (% edema inhibition) | Paw edema volume (3 hrs) Mean + SEM (% edema inhibition) | Paw edema volume (4 hrs) Mean + SEM (% edema inhibition) | Paw edema volume (5 hrs) Mean + SEM (% edema inhibition) | Paw edema volume (6 hrs) Mean + SEM (% edema inhibition) |
|---|---|---|---|---|---|---|---|
| 1 | NC | 0 | 0 | 0 | 0 | 0 | 0 |
| 2 | DC | 1.01+0.02 | 0.95+0.03 | 0.88+0.04 | 0.85+0.03 | 0.84+0.01 | 0.82+0.03 |
| 3 | SC | 0.63+0.02 (28.41%) | 0.53+0.01 (37.64%) | 0.58+0.03 (38.92%) | 0.35+0.01 (65.34%) | 0.28+0.03 (72.27%) | 0.31+0.02 (62.19%) |
| 4 | TC | 0.71+0.02 (16.43%) | 0.68+0.03 (22.72%) | 0.37+0.02 (57.95%) | 0.18+0.02 (81.05%) | 0.14+0.02 (83.33%) | 0.12+0.03 (85.36%) |

**Table 8:** Paw edema volume (ml) and percentage edema inhibition in different treatment groups (Mean+SEM)

NC: Normal Control, DC: Disease control, SC: Standard Control, TC: Test Control Each value represents the mean of 6 animals + standard error of the mean. Statistical analysis was determined using a one-way ANOVA test. All groups differ significantly ($p<0.05$) from DC group.

## 4. CONCLUSIONS

The present study aimed at formulation, characterization and evaluation of anti- inflammatory effects of LFs of CURC in combination with ETRX in experimental AI in rat model induced by carrageenan administration.

CURC and ETRX loaded LPs were prepared by thin film hydration method followed by probe sonication using PC, PL and CL. The present study evaluated the anti-inflammatory effects of conventional CURC and ETRX and CURC-ETRX LFs in experimental carrageenan induced inflammation in paw edema in rat model. The results of our study demonstrated anti-inflammatory formulation to reduce paw edema at low dose in rat models; also the release profile was sustained up to 6 hours in comparison to 4 hours release profile achieved with conventional formulations. It is concluded that by reducing CURC particle size to the nanometer size range in the form of LFs, the therapeutic efficacy is increased. Hence, the synergistic effect of CURC and ETRX in the form of nanosized LF could potentiate the anti-inflammatory effects in reduced doses in rat model.

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