Nanoparticles Loaded Gels for Topical Delivery of Curcumin and Celecoxib for the Treatment of Osteoarthritis

M.Phil. Thesis

by

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Nanoparticles Loaded Gels for Topical Delivery of Curcumin and Celecoxib for the Treatment of Osteoarthritis

Thesis Submitted by

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DEDICATION

He intends a thing that He says to it, "Be", and it is.

(Al Qur'an 2; 117)

Indeed, without the will and help of almighty Allah there is no possibility of a task initiating, let alone reaching its completion.

I would like to dedicate this piece of work to my noble discipline of pharmacy. I hope that someday this work might be of some benefit for the generations to come.

My second and most important dedication is to my mother and daughter without whose patience and resilience I could never have achieved this pinnacle of success.

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List of Abbreviations

Abbreviation	Description
OA	Osteoarthritis
IL-1,6	Interlukin 1 and 6
TNF-α	Tumour necrosis factor-alpha
NSAIDs	Non-steroidal anti inflammatory drugs
DMOADs	Disease modifying osteoarthritic drugs
NF-κB	Nuclear factor kappa B
BCS	Biopharmaceutical classification system
COX	Cyclooxygenase
PLGA	Polylactic co-glycolic acid
FDA	Food and drug administration
PLGA-cap	Polylactic co-glycolic acid calcium phosphates
CP	Carbopol
PVA	Polyvinyl-alcohol
TEA	Triethanolamine
NPs	Nanoparticles
SEM	Scanning electron microscope
PBS	Phosphate buffer saline
EA	Ethyle acetate
PDI	Polydispersity index
ANOVA	Analysis of variance
$Na_2HPO_4.2H_2O$	Disodium hydrogen phosphate
KH_2PO_4	Potassium di-hydrogen phosphate
NACL	Sodium chloride
HCL	Hydrochloric acid
NaOH	Sodium hydroxide
CFA	Complete freunds adjuvant
mm^2	Millimeter square
µl	Microliters
JSS	Steady state flux
KP	Permeation co-efficient
siRNA	Short interfering ribonucleic acid

SPIONs	Super paramagnetic iron oxide nanoparticles
GIT	Gastrointestinal Tract

ABSTRACT

Osteoarthritis (OA) is known as the third leading cause of physical disability due to its high prevalence, specifically among the elderly population. Curcumin is a drug of natural origin which has marked anti-inflammatory activity while Celecoxib is one of the drugs prescribed as first line treatment for osteoarthritis. Both of these drugs have a reported synergistic anti-inflammatory activity. Therefore, it was considered important to combine these two drugs in a single nano formulation to further improve their efficiency and to minimize their associated toxicities. For this purpose, study was designed to prepare and characterize Curcumin – Celecoxib loaded Poly lactic co glycolic acid (PLGA) nanoparticles by oil in water (O/W) emulsion solvent evaporation technique. The prepared nanoparticles were then loaded into Carbopol-934 (CP-934) gel for topical application in OA. The nanoparticles were characterized for their size, size distribution, morphology, encapsulation efficiency and invitro release profile. Nanoparticles in the size range of 150 to 220 nm were successfully fabricated. A distinct spherical shape of nanoparticles was revealed in scanning electron microscopy (SEM). The drug release profile indicated approximately 80% release of both drugs from nanoparticles within 48 hours via Fickian diffusion mechanism. The CP-934 gels loaded with nanoparticles were also characterized for their spreadability, viscosity, stability and permeability studies. All the in-vitro characterizations demonstrated that the co-delivery of Curcumin and Celecoxib in PLGA nanoparticles loaded in CP-934 gel serve as an efficient carrier system for targeted delivery in Osteoarthritis. The drug content of both drugs in final gel formulation was approximately between 70% to 85% for both drugs. In addition, preliminary *in-vivo* studies were conducted on albino mice for therapeutic effectiveness of the prepared nano formulation.In this connection behavioral tests were conducted and statistically significant results with p-value <0.05 were obtained in the group of animals treated with PLGA nanoparticle loaded gels of Curcumin and Celecoxib. Furthermore, detailed *in-vivo* studies will be essential to completely understand the synergistic mechanism of these two drugs in relevant animal models.

CHAPTER 1

INTRODUCTION

1. Introduction:

1.1 Osteoarthritis

Osteoarthritis (OA) is a degenerative, chronic inflammatory disorder which affects various joints of the body. A number of etiologies are considered to cause this disease, however there is still a lack of availability of safe long-term treatment .OA is known to cause disability and constricted mobility in elderly population due to its progression (Lev-Ari *et al.*, 2005b). Pain and physical dysfunction are two of the most important symptoms of OA. The symptomatic clinical presentation of osteoarthritis varies from, patient to patient as there are a number of factors responsible for the onset and progression of this disease (Calders and Van Ginckel, 2017). Some etiological factors involved in the onset of this disease may include external pressure (trauma), abnormal stress on joint surfaces, genetic predisposition, injury and hormonal alterations (Figure 1.1). Although it is difficult to pinpoint the exact biochemical cause of osteoarthritis, pain which aggravate with movement, stiffness of the joint, and physical immobility are usually the reported symptoms (Reddy and Faruqui, 2016). Risk factors that may lead to development of OA include, gender, ethnicity/race, hormonal alterations, obesity which might cause abnormal stress on the joints. Some notable risk factors include gender, race/ethnicity, bone density, postmenopausal loss of estrogen, and nutritional factors. Some biomechanical risk factors include obesity, quadriceps muscle weakness, joint injury, joint trauma, and malformation (Ashford and Williard, 2014).

Figure 1.1. Etiology of OA.

1.1.1 Epidemiology of OA:

Due to high prevalence of OA, it is considered to be one of the major health problems in the world. The slow progression and chronic nature of this disease is known to reduce quality of life of patients significantly. Incidence of OA is highest in American Indians and women throughout the world. Even though the underlying causes are not yet comprehended, it is more prevalent in women than in men after the age of 55. Incidence of OA is found to be directly proportional to age of patient, however it is not an inevitable consequence of aging. Regardless, age still remains the strongest predictor (Ashford and Williard, 2014). It is characterized as third leading cause of life-years lost due to disability. Prevalence of this disease ranges from 38.1 to 46.8% among Asian countries (Kuptniratsaikul *et al.*, 2014).

1.1.2 Pathogenesis of OA:

In OA as the disease progresses the complete joint structure is affected. The articular cartilage is destroyed due to which the bone structure is altered. Synovial membrane becomes inflamed and inflammation of the hyaline cartilage occurs. This cartilage structure, under normal physiological conditions has the function of distributing large loads and reducing friction in the joints. However the inflammation of hyaline cartilage increases joint friction and lack of joint space occurs which further increases stress on joints (Ashford and Williard, 2014). An ongoing inflammatory reaction is triggered with the breakdown of cartilage matrix which causes migration of macrophages and neutrophils at the site of inflammation. Ulcerations are formed as a result of this inflammation and bone erosion occurs. Proinflammatory cytokines are responsible for catabolic process occurring in pathological tissues. The synovial membrane produces these cytokines and when they diffuse into the cartilage through synovial fluid. The synovial lining cells called chondrocytes are activated. Inflammatory markers such as interleukin 1 (IL-1) and interleukin 6 and Tumor necrosis factor-alpha (TNF-alpha) are considered the main catabolic pathways for cartilage destruction in OA (Martel-Pelletier, 1999). Whenever there is inflammation in the body, presence of new blood vessels or angiogenesis occurs due to enhanced cell proliferation. Both of these processes are closely integrated. The disease progression and pain in OA may be affected by the process of angiogenesis. Inflammation can stimulate angiogenesis which in turn can stimulate more inflammation hence an ongoing cycle of chronic inflammation begins (Sellam and Berenbaum, 2010).

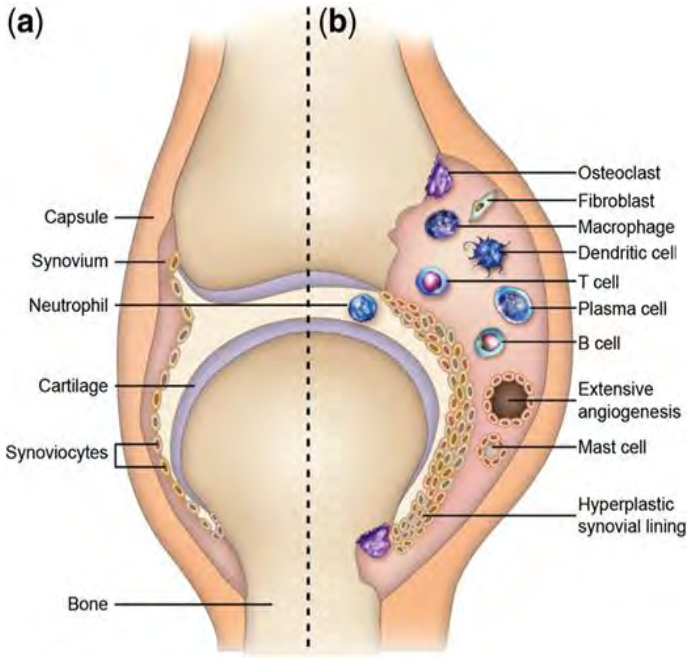


Figure1.2. comparison between a normal and arthritic joint ;(a):normal joint,(b):arthritic joint.(Choy, 2012)

1.2 Treatment strategies for OA:

The treatment strategies for OA can be broadly categorized into three groups. Life style modification, pharmacological treatment and finally invasive surgical procedures. The main aim of this treatment is to provide symptomatic relief from pain and inflammation (Barnes and Edwards, 2005). Table 1.1 gives brief overview of treatment strategies

Table 1.1 Treatment strategies for OA

Non-Pharmacological Treatment	Pharmacologic Treatment	Invasive Procedures
Weight loss Exercise Diet modification Knee braces Occupational gastrointestinal therapy	NSAIDs Analgesics Glucosamine and chondroitin sulfate DMOADs Corticosteroids Opioid analgesics	Orthopedic surgery Debridement of joint Arthroplasty

1.2.1 Problems associated with current treatment available for OA:

Apart from non-pharmacological treatment, non-steroidal anti-inflammatory drugs (NSAIDs) and analgesics are the primary choice of drug as a part of standard care in OA (Reddy and Faruqui, 2016). Although these drugs are effective in management of pain, fever and edema which are the symptomatic consequences of inflammatory mediator release, their long term use is associated with gastrointestinal, hepatic and renal insufficiencies (Suleyman *et al.*, 2007). Although the primary pharmacological treatment does provide symptomatic relief in an efficient manner, it exerts no effect whatsoever on disease prevention or modification (Zhang *et al.*, 2016).

Corticosteroids are also prescribed orally and parentally for management of this condition. However their chronic use is associated with many adverse effects including osteoporosis, aseptic joint necrosis, adrenal insufficiency, gastrointestinal, hepatic, and ophthalmologic effects, hyperlipidemia, growth suppression, and possible congenital malformations (Buchman, 2001). Even supplements such as glucosamine are associated with gastrointestinal adverse effects on oral use (Müller-Faßbender *et al.*, 1994). Opioid analgesics have adverse effects such as constipation and nausea since they are centrally acing analgesics (Furlan *et al.*, 2006).

Table1.2. limitations of current therapy of OA

Pharmacological Treatment	Route of Administration	Adverse Effects	References.
NSAIDS	Oral and Topical	G.I, Hepatic, Renal Toxicities	(Suleyman, Demircan *et al.* 2007
Glucosamine	Oral	G.I, Nausea Diarrhea	(Müller-Faßbender *et al.*, 1994)
Corticosteroids	Oral and Injectables	Osteoporosis, Hyperlipidemia G.I Adverse Effects, Obesity	(Buchman, 2001)
Opioid Analgesic	Oral and Injectables	Decreased Patient Compliance	(Furlan *et al.*, 2006)
DMAODs	Oral and Injectables	Bone Marrow Suppression, Hepatotoxic, Cardiotoxic	(Simon, 2000)

1.2.2 Comorbidities; an added complication:

Approximately 15% population of individuals of both genders above 60 years of age have been found to be affected by OA. Presence of co-existing medical problems is very common in patients of OA since the progression of this disease is directly proportional to age. Hall *et al.* has reported cardiovascular disease was co-existing alongside OA in approximately 40% of patients. Some other co-existing medical conditions commonly present in patients of this disorder include obesity, diabetes, hypertension, depression and backache (Calders and Van Ginckel, 2017). All the medical conditions that exist alongside arthritis in patients of old age are the reason of in-tolerance to adverse effects as well as drug interactions in arthritic patients.

1.3 Nanocarrier based topical/transdermal treatment of OA:

The treatment of chronic musculoskeletal disorders such as OA could benefit from topical administration for a number of reasons, including marked reduction in systemic side effects and an improvement of the patient compliance. However, skin serves as the first line of defense and a significant barrier in order to protect the tissues and deeper muscles of the body (Puglia *et al.*, 2008). While topical or transdermal drug delivery provides the benefit of bypassing the first pass effect, the barrier of skin needs to be exploited in order to achieve drug permeation across the skin into the muscles. Therefore, the use of nanocarriers is one of the most promising drug delivery system across the skin (Bouwstra and Honeywell-Nguyen, 2002). A number of systems such as polymeric nanoparticles, lipid nanoparticles, liposomes and others are included in nanocarriers. The small size of the nanoparticulate carriers have been suggested as the reason such vehicles can enhance the skin permeation of the pharmaceutical active ingredients. They entrap, while at the same time protect pharmaceutical active ingredient from degradation, while providing the benefit of sustained release of drugs (Elmowafy *et al.*, 2017).

1.4 Curcumin and Celecoxib nanoparticles- a novel approach to treat OA:

In the current scenario there is a research gap in proper, long-term treatment of OA which has improved effectiveness, improved patient compliance and lesser side effects. Keeping these outcomes in mind, the current study focuses on development of PLGA nanoparticles co-loaded with curcumin and celecoxib for local application on arthritic joints.

1.4.1 Curcumin as DMAOD:

Curcumin a yellow pigment isolated from the rhizomes of Curcuma longa, commonly known as turmeric, is a highly pleiotropic molecule with an excellent safety profile. Strong evidence of the molecular level has been published for its potency to target multiple inflammatory diseases (Huang *et al.*, 1991). Curcumin was found to inhibit the formation of prostaglandins through cyclooxygenase and lipoxygenase pathway (Rao *et al.*, 1995).

Several recent in vitro studies provide evidence which suggests that chondroprotective effect through actions such as anti-inflammatory, anti-oxidative stress, and anti-catabolic activity which are critical for progression of OA, may be exerted by curcumin. It has been shown to mitigate the inflammatory process by decreasing synthesis of inflammatory mediators such as interleukin (IL)-1β, tumor necrosis factor (TNF)-α, IL-6, IL-8, prostaglandin E2 (PGE_2), and cyclooxygenase-2 (COX-2) (Goel *et al.*, 2001).

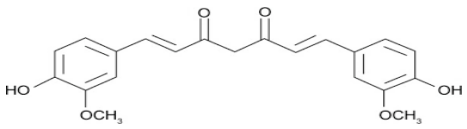


Figure 1.3. chemical structure of curcumin

However oral administration of curcumin has the limitation of poor bioavailability as it is a poorly soluble hydrophobic compound (Rajasekaran, 2011). Chondrocytes were treated with curcumin and suppression of IL-1b-inducedNF-kB activation was observed. It was found that curcumin inhibited IL-1b induced stimulation of protein kinases. Incidentally down regulation of COX-2 and metalloproteases was observed due to curcumin. It was hence concluded that curcumin has nutritional potential of treating OA through suppression of NF.KB mediated IL.1B and TNF catabolic pathways in chondrocytes (Shakibaei *et al.*, 2007). The dietary effect of non-steroidal anti-inflammatory drugs (NSAIDs) or curcumin was studied on the gene expression of white blood cells (WBCs) in canines effected by OA. It was found that after administration of curcumin for 20 days the genes involved in inflammatory response dramatically decreased in the arthritic dogs. Inhibition of macrophages proliferation and strong down regulation of TNF .alpha was also observed due to curcumin (Colitti *et al.*, 2012). Curcumin was found to significantly slow OA disease progression and exerted a disease modifying effect (Zhang *et al.*, 2016).

Curcumin is a highly hydrophobic and according to Biopharmaceutical Classification system (BCS) it is a class four drug. The problems associated with it include low solubility

and low permeability. Since this drug possesses herbal origin it can be prescribed in higher doses with minimal risk of side effects. It is extensively studied in case of inflammatory conditions and various other disorders.

1.4.2 Celecoxib -first line of therapy for arthritis:

Celecoxib is a drug belonging to BCS class II. It has poor solubility and high gastric permeability. It is a selective cyclooxygenase two (COX-2) inhibitor. This drug is prescribed for the management of pain and inflammation is rheumatic conditions and OA. It is equivalent to NSAIDs in case of symptomatic relief from pain and inflammation but with the added benefit of fewer GI related side effects. However prolonged use of celecoxib in chronic inflammatory conditions causes cardiovascular risk (Silverstein *et al.*, 2000). Since the vascular GI effects of NSAIDS including selective COX-2 inhibitors are not well characterized, these medicines should be used with caution in patients already at risk for vascular disease (Bhala *et al.*, 2013).

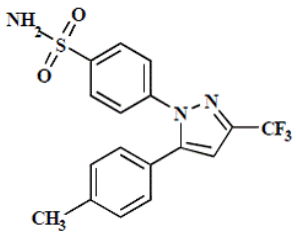


Figure 1.4. chemical structure of celecoxib

Celecoxib also possesses anti-cancer properties. The selective inhibition of COX-2 is thought to lead to a reduction in the unwanted effects of NSAIDs. It is mainly metabolized through cytochrome p-450 isoenzyme. However, in elderly patients changes in pharmacokinetics of this drug have been reported. Renal insufficiency causes the plasma concentrations of celecoxib to lessen by 43% when compared to healthy individuals (Davies *et al.*, 2000). Celecoxib is also known to inhibit angiogenesis , by inducing endothelial apoptosis and therefore possesses a marked anti-inflammatory effect (Raut *et al.*, 2004).

1.4.3 Synergistic anti-inflammatory action of combined use of Curcumin and Celecoxib:

Celecoxib is a COX-2 inhibitor whereas curcumin is COX-1 and COX-2 inhibitor. The synergistic action of these drugs used in combination has been reported a number of times

in the case of OA, inflammatory bowel syndrome, as well as cancer (Lev-Ari *et al.*, 2005a, Gugulothu *et al.*, 2014, Lev-Ari *et al.*, 2005b). The literature has enough evidence of oral administration of curcumin and celecoxib or parenteral administration of both these compounds or each, there is so far no study reported which utilizes the synergistic action of these compounds topically for the treatment of chronic inflammation such as in arthritis. Figure 1.5 is the schematic diagram describing how curcumin and celecoxib can inhibit inflammation in the case of OA.

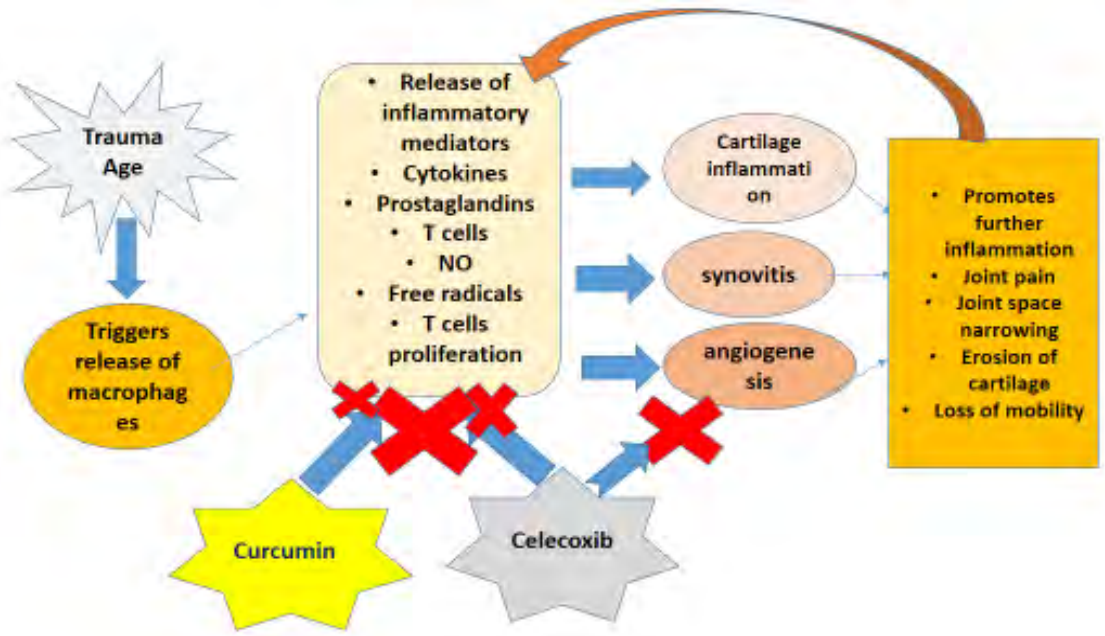
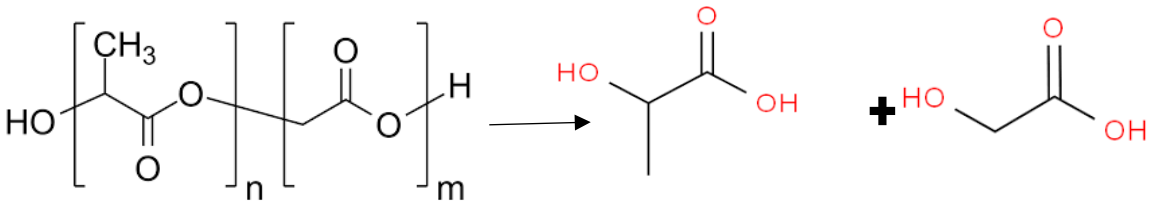


Figure 1.5. schematic representation of role of curcumin and celecoxib in preventing inflammation in arthritis.

1.5 Polylactic co-glycolic acid- benefits of use in polymeric system designed for OA:

Poly lactic co glycolic acid (PLGA) is an FDA approved polymer and has been one of the most attractive candidates for fabricating devices for drug delivery in the past two decades. It is known to possess adjustable mechanical properties therefore exhibits a wide range of erosion times (Makadia and Siegel, 2011). PLGA is biodegradable because it undergoes hydrolysis in the body to produce metabolites which are biodegradable such as glycolic acid and lactic acid (Fig 1.6). Since both of these metabolites are effectively dealt by the

body, there is very minimal risk of toxicity caused due to the use of this polymer (Kumari *et al.*, 2010). Figure 1.6. illustrates the metabolites of PLGA in the body.



PLGA LACTIC ACID GLYCOLIC ACID

Figure 1.6. metabolites of PLGA

Polylactic acid-co-glycolic acid-calcium phosphates (PLGA-CaP) are extensively used in orthopedic applications (Zhou *et al.*, 2012). Microspheres fabricated through PLGA were prepared by a modified method of solvent evaporation. These microspheres when administered intra articular, slowly release siRNA and effectively inhibited expression of TNF-alpha in arthritic joints (Présumey *et al.*, 2012) .

In another study steroidal treatment for chronic inflammatory conditions, such as arthritis was achieved using PLGA. Prednisolone-loaded microspheres using PLGA were prepared using o/w emulsion solvent evaporation method. Sustained release of drug was achieved through this polymer. Further animal testing confirmed the marked reduction in inflammatory edema in complete freund's adjuvant (CFA) injected mice. This concluded successful delivery of the drug with the help of polymeric nanoparticles (Khaled *et al.*, 2010). Superparamagnetic iron oxide nanoparticles (SPIONs) and corticosteroid dexamethasone acetate were co encapsulated into PLGA for locally treating arthritis and other inflammatory conditions (Butoescu *et al.*, 2009) .

1.5.1 Selection of method to fabricate nanoparticles:

There are a number of methods used to prepare PLGA nanoparticles. Solvent emulsion-evaporation, interfacial deposition, solvent evaporation and nano-precipitation techniques are among the most popular (Kumari *et al*., 2010).

Although all of the above mentioned techniques can be employed for fabricating PLGA nanoparticles. The most common method used for the preparation of solid, polymeric

nanoparticles is the emulsification-solvent evaporation technique. Hydrophobic drugs are primarily encapsulated through this method. For hydrophilic drugs some modifications of this procedure are used such as double or multiple emulsion technique (Makadia and Siegel, 2011). Since both curcumin and celecoxib are hydrophobic in nature, therefore emulsification solvent evaporation method is utilized in this study to encapsulate both drugs.

1.5.2 Selection of route of administration:

The topical route of drug delivery is a vast term and can be used to describe a number of dosage forms. Drug delivery through the skin is complex due to the complex structure of stratum corneum. However, in acute and chronic pain and inflammation topical non-steroidal anti-inflammatory drugs remain effective in relieving pain due to patient compliance and ease of application (Moore *et al.*, 1998). Utilizing the topical route helps by-pass the adverse effects associated with oral or systemic drug delivery (Mundada *et al.*, 2012).Vesicles and particles of the nano range such as liposomes, transferosomes, and niosomes provide the benefit of deeper penetration into the layers of stratum corneum due to lesser size (Barry, 2001). Topical drug delivery system using nanospheres and Nano capsules was designed for indomethacin. It was found that resultant formulation had higher pharmacodynamic effect as compared to the marketed formulation of the drug (Elmowafy *et al.*, 2017). Nanoparticles having diameter within the range of 20-200nm are known to successfully penetrate within the skin and their distribution within the skin increases in a time dependent manner. The localization of nanoparticles within the stratum corneum burrows is favored by the smaller particle size (Alvarez-Román *et al.*, 2004).

1.5.3 Selection of final dosage form:

The topical route of drug delivery can be broadly classified into three major categories based on the state and different properties of each category. These are solids such as dusting powders, liquids such as lotions and liniments and semi solids such as creams, ointments, pastes and gels. Pharmaceutical excipients which are the non-drug component of dosage form are used in dosage form as inactive ingredients or as tools for structuring dosage forms (Garg *et al.*, 2015). Among these, the semi-solids gels provide the benefit of providing faster release of drug substance irrespective of the water solubility of the drug. Gels have lower risk of inflammation and high biocompatibility with less adverse effects. For topical administration of drugs, gels provide several favorable properties such as spreadability,

thixotropic behavior, water solubility, greaseless application, easy application and ease of removal (Helal *et al.*, 2012). Since both curcumin and celecoxib have the limitation of decreased water solubility, therefore formulating them into a gel matrix will provide with better drug release on skin.

1.5.4 Carbopol-934 for formulation of nanoparticles loaded gel:

Carbopol-934 (CP) is a synthetic polymer made of carbomers. CP is extensively used to formulate pharmaceutically elegant gels due to its property of cross linking. Cross linking which is found in carbomer polymers helps in the formation of a microgel structure. Since carbomers are highly anionic in nature, addition of a strong base such as triethanolamine for the purpose of neutralization is necessary in order to obtain gel structure (Islam *et al.*, 2004).

It possesses good mucoadhesive strength and stability (Tamburic and Craig, 1995). Due to good thixotropic properties , highly viscous and transparent gels can be formulated with CP even at low concentrations (Gupta *et al.*, 2010). Permeation enhancers are also utilized in various studies in order to increase the permeation of CP based gels across the skin (Mundada *et al.,* 2012). Gels of diclofenac sodium formulated with CP and propylene glycol, a permeation enhancer showed better permeability and permeation flux as compared to gels formulated with other polymers (Talele *et al.*, 2017).

1.6 Problem statement:

A chronic and progressive disorder like OA has the added complication of co-morbidities present in the patients of old age. Therefore, there is a need for dosage forms, designed to provide maximum anti-inflammatory activity while maintaining patient compliance, and reducing the incidence of adverse drug reactions.

1.7 Rationale of the study:

The rationale of this study can be summarized as the following:

Patients presenting with osteoarthritis have co-existing medical conditions due to age and chronic use of drugs. Therefore, it is necessary to design treatment strategies that minimize adverse effects and provide the necessary relief from immobility and pain in a patient friendly way. Curcumin and Celecoxib have reported synergistic anti-inflammatory action in a number of inflammatory conditions however both the drugs have poor solubility. In

this scenario combining the PLGA nanoparticles of these two drugs in a single formulation as patient friendly gel is the aim of this study. In order to provide better skin penetration, PLGA nanoparticles provide the benefit of smaller size therefore deeper penetration within the layers of the skin and deeper tissues can be achieved. Furthermore, these nanoparticles are formulated into CP gels for easy application on the skin and joints.

1.8 Aim of the study:

The main aim of this study is to develop PLGA nanoparticles co-loaded with curcumin and celecoxib and fabricate these into a CP gel for sustained anti-inflammatory action as well as improved retention in arthritic joints when applied.

1.9 Objectives:

The objectives include:

- Fabrication of PLGA nanoparticles of curcumin and celecoxib.
- Optimization and characterization of nanoparticles.
- Formulation of drug loaded nanoparticles as CP gel.
- Optimization and characterization of gels.
- Study of release pattern of both drugs from nanosuspension and nanoparticles loaded gel.

CHAPTER 2

MATERIALS AND METHOD

2. MATERIALS AND METHOD

2.1 Chemicals:

Poly lactic co-glycolic acid (PLGA) 50:50 polymer (Resomer RG 502) was obtained by Sigma Aldrich,Germany. Curcumin was gifted by Ijaz ul haq, University of Swabi. Celecoxib was obtained by United Pharmaceuticals.co Germany. Polyvinyl Alcohol (Mowiol) MW 31000 was received from Sigma Aldrich, Germany. Ethyl acetate, Methanol and Hydrochloric Acid (HCl) and tri-ethanolamine (TEA) were purchased from BDH laboratory Supplies, England. CP-934 was obtained by Avonchem United kingdom. t. Disodium hydrogen phosphate ($Na_2HPO_4.2H_2O$), Potassium di-hydrogen phosphate (KH_2PO_4) and Sodium chloride (NaCl) for the preparation of phosphate buffer solution were also procured from BDH laboratory Supplies.

2.2 Equipment and Apparatus:

Weighing balance (Ohaus corporation, PA 214C, USA), Hot plate Multi Stirrer (MAGIK, MG-855), Water distillation apparatus (IRMECO GmbH IM50, Germany), Centrifuge Machine (Hermle, GmbH Z326k, Germany), Agilent 8543 Ultraviolet-Visible Spectrophotometer (Agilent technologies, United States), Bath sonicator (Elmasonic GmbH, E60H, Germany), Probe sonicator (Misonix- XL-2000 series), PH meter (Bante instruments, PHS 25GW, Chicago, USA), Refrigerator (Panasonic, MPR-161D H, Japan), Biomedical freezer (Panasonic, MDF-137, Japan), Oven (Memmert, INB200, Germany), Lyophilizer (Christ Alpha 1-2 LD plus, Germany), Perkin-Elmer spectrum 100 FTIR spectrometer (US), Scanning Electron Microscope (SEM) (VEGA3, Tescan), Malvern Zeta sizer.

2.3 Methods:

2.3.1 Preparations of solutions:

2.3.1.1 2% P.V.A solution:

2 grams of poly vinyl-alcohol (PVA) was carefully weighed. A small amount of PVA was added in 50ml of distilled water in a beaker while stirring . Gradually the remaining amount of PVA was added and the volume was made up to 100ml. This solution was heated in a bath sonicator for 45 minutes at 60C. Then it was left for overnight stirring at room temperature.

2.3.1.2 Preparation of phosphate buffer saline (PBS) solution:

Phosphate buffer saline (PBS) was prepared by dissolving Na_2HPO_4 (238 mg), KH_2PO_4 (19mg) and Nacl (800mg) in 100ml of distilled water PH was adjusted with the help of diluted HCL and NaOH.

2.3.1.2.1 Calibration curve in methanol:

The solubility of both the drugs was checked in methanol, ethanol and acetone. It was observed that curcumin and celecoxib exhibited no precipitation in methanol. Therefore,1 mg of each drug was dissolved in 100ml methanol in order to make stock solution of concentration 10ug/ml.

From this stock solution 1ml was withdrawn each time in order to make further dilutions of known concentration. The volume required of the methanol for each concentration was calculated by;

$C^1V^1=C^2V^2$

Where;

C^1 = Concentration of stock solution V^1 =Volume of stock solution

C^2 =Concentration of succeeding dilution V^2 =Final volume of succeeding dilution

Table 2.1 dilutions of calibration curve in methanol

Concentration of dilution	Volume of methanol
0.2ug	50ml
0.4ug	25ml
0.6ug	16.66ml
0.8ug	12.5ml
1ug	10ml
1.2ug	8.33ml

These concentrations were checked for absorbance on UV visible double beam spectrophotometer at lambda max 252 for celecoxib and 421 for curcumin. Absorbance value against each dilution was recorded to plot a graph against respective dilution concentration and R^2 value was obtained along with linear regression equation, given by;

$$y = mc + x$$

Where y is the absorbance at λ max, m denotes the slope; x is the unknown concentration of the drug in mcg/ml and c represents the intercept.

To calculate drug loading and encapsulation efficiency of drug in nanoparticles equation was rearranged as follow,

$X = \frac{y - c}{m}$

2.3.1.2.2 Calibration curve in phosphate buffer PH 5.5:

PBS was prepared as method previously reported by dissolving Na_2HPO_4, KH_2PO_4 and NaCl in a specific quantity of water (2.3.1.2).

The PH was adjusted to 5.5 using diluted HCL. The stock solution of both drugs was prepared in methanol in the concentration of 10ug/ml (1mg of methanol). Further dilutions were made for various concentrations using only 1ml of the stock solution each time (Table 2.2).

Table 2.2 dilutions of calibration curve in PBS 5.5

Concentration of dilution	Volume of buffer PH 5.5
0.6	16.66ml
0.8	12.5ml
1.0	10ml
1.2	8.33ml
1.4	7.14ml
1.6	6.25ml
1.8	5.55ml
2.0	5ml

2.3.1.2.3 Calibration curve in phosphate buffer PH 7.4:

The methodology of making calibration curve in phosphate buffer 7.4 PH is exactly the same as described previously for phosphate buffer with PH 5.5 (2.3.1.2.2).

2.4 Preparation of Nano formulation:

2.4.1 Preparation of blank nanoparticles:

Blank nanoparticles were prepared by modified single emulsification solvent evaporation method (Kim and Martin, 2006). For this purpose, 2.5ml EA (Ethyl Acetate) was taken and 50mg of PLGA was dissolved in it. This solution was then injected into 2% PVA (Poly Vinyl Alcohol) while stirring. This emulsion was left to stir for two hours and then sonicated for 1 minute at voltage 4. After this, the solution was left on stirring overnight for complete evaporation of solvent.

2.4.2 Preparation of drugs loaded nanoparticles:

2.5ml of ethyl acetate was taken in which 50mg of PLGA was dissolved, then both the drugs Celecoxib and Curcumin were weighed as 5mg each drug and added into the EA mixture. After both drugs were dissolved, this solution was injected dropwise into 2% PVA and left to stir for two hours. After two hours it was sonicated for one minute through probe sonicator and was left to stir overnight while covered with a foil which had holes made in it in order to evaporate the organic solvent (Figure 2.1).

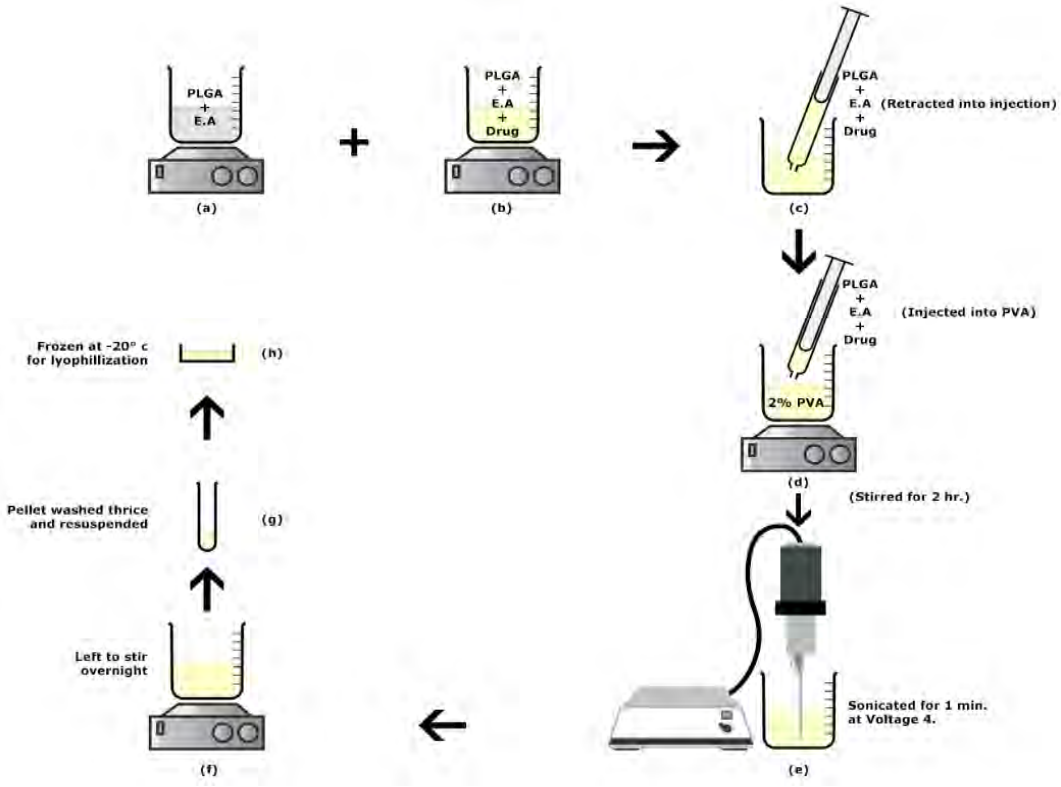


Figure 2.1 schematic diagram of oil in water solvent evaporation technique to prepare drug loaded nanoparticles

2.4.2.1 Parameters studied and adjusted:

The process parameters which were studied and adjusted accordingly included stirring speed, surfactant concentration and drug concentration (Makadia and Siegel, 2011). The desirable size of nanoparticles for topical formulations which facilitates deeper penetration into skin and tissues is between 20nm too 200nm (Alvarez-Román *et al.,* 2004). This is why the Nano formulations were optimized while keeping some desirable outcomes in mind (Figure 2.2).

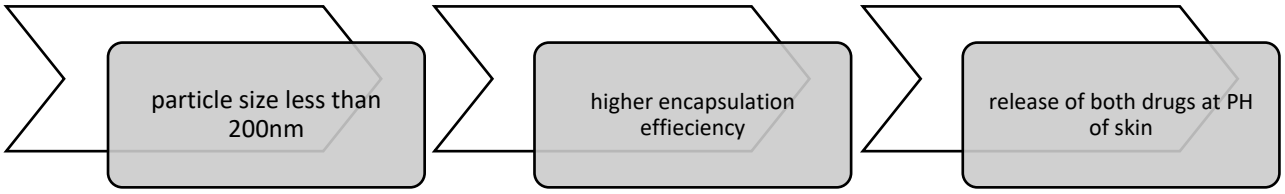


Figure 2.2 Desirable outcomes of Nano formulation

Table 2.3 summarizes the parameters adjusted for the Nano formulation:

Table 2.3 Summary of parameters adjusted

Form.	E: A to PVA ratio	Drug content	% of PVA solution
A.1	1:1	Blank	2%
A.2	1:2	Blank	2%
A.3	1:3	Blank	2%
B.1	1:1	5mg each	2%
B.2	1:1	7.5mg each	2%
B.3	1:1	10mg each	2%
C.1	1:1	7.5mg each	1%
C.2	1:1	7.5mg each	1.5%
C.3	1:1	7.5mg each	2%

2.4.2.2 Freeze drying of Nano formulations

Curcumin and celecoxib loaded PLGA Nano formulation were first centrifuged at 13,500rpm for 30 min. Supernatant was collected for analyzing indirect encapsulation efficiency and the pellet was washed thrice with distilled water for proper removal of unentrapped drug. To purified pellet 3ml of distilled water was added, dispersed it and

incubated at -80°C for 24 hours. Afterwards the frozen samples were lyophilized by using Christ alpha 1-2 LD plus Lyophilizer, to get solid nanoparticles and stored at 4°C for later use.

2.5 Preparation of nanoparticles loaded CP-934 gel:

To prepare nanoparticles loaded CP gel the basic method included weighing a specific amount of CP and dissolving it in water. Once the CP was fully dissolved in water, stirring was continued for two hours to allow proper hydration of CP solution. After this the nanoparticle pellet which was previously separated from the nanosuspension was added in the CP solution. This mixture was stirred for one hour to form a homogenous mixture. Afterwards a few drops of TEA (Triethanolamine) was added and gel formation occurred (Jana *et al.*, 2014) (figure 2.3).

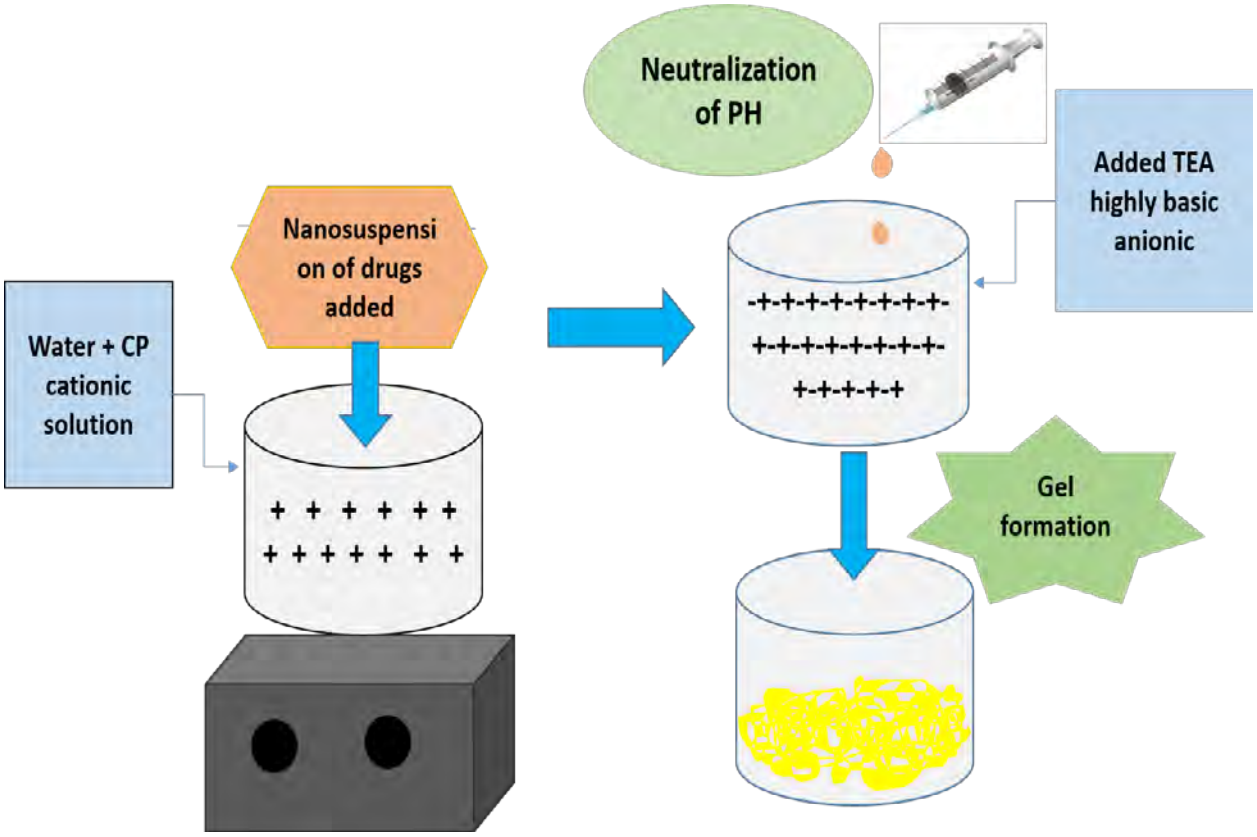


Figure 2.3 preparation of CP gel

2.5.1 Optimization of nanoparticles loaded CP gel:

Table 2.4 depicts the optimization of nanoparticles loaded CP gel. Different concentrations of CP were made, gel from each concentration was formulated and checked for various desired outcomes.

Table 2.4 Optimization of CP gel.

Weight of CP (mg)	Vol. of water added	Concentration of CP solution
125mg	5ml	2.5%
100mg	5ml	2%
75mg	5ml	1.5%
50mg	5ml	1%
25mg	5ml	0.5%

The number of drops of T.E.A depended upon the concentration of CP solution. It was observed that the more concentrated the CP solution was, the less amount of triethanolamine was needed to form gel.

2.6 Characterization of Nano formulation:

2.6.1 Particle size analysis:

Zeta sizer and Scanning electron microscopy (SEM) were used to study particle size. Particle size analysis was done in triplicate and the results were expressed in mean ± SD (standard deviation).

2.6.2 Polydispersity index (PDI):

Molecular weight distribution or homogeneity in the particle size distribution was estimated by PDI with the ideal range from 0-1. Lower the value, more mono-dispersity in the system exists. PDI of Nano system was analyzed by Zeta sizer.

2.6.3 Surface morphology of nanoparticles:

In order to study the structure and morphology of nanoparticles SEM was used. To prepare sample for analysis in SEM a tiny drop of diluted NPs sample was poured on a glass slide. It was then dried in drying oven at 60° C for 10 minutes, coated with gold through gold sputtering and analyzed under SEM.

2.6.4 Zeta potential:

For stability of Nano emulsion, presence of charge is favorable and zeta potential dictates about this charge. Zeta potential is an electro kinetic parameter which prevents the aggregation of particles and keeps the particles dispersed by creating a least repulsive force. Zeta potential of Curcumin-Celecoxib loaded PLGA NPs was measured by Malvern Zeta sizer.

2.6.5 Percent encapsulation efficiency:

Curcumin and celecoxib entrapped within the nanoparticles was determined by indirect method. Indirect drug loading was determined by collecting the supernatant after washing the nanoparticles thrice with distilled water. Absorbance values obtained from UV-Visible double beam spectroscopy at 421 λ max for Curcumin and 252 λ max for Celecoxib were used to analyze the free drug content in the supernatant and % encapsulation efficiency was calculated by formula below;

$$\text{Entrapment efficiency} = \frac{total\ amount\ of\ drug - free\ drug}{total\ amount\ of\ drug\ loaded} * 100$$

2.6.6 Fourier transform infrared (FTIR) spectral analysis:

The chemical composition of the samples was analyzed by FTIR spectroscopy. PLGA, Curcumin -Celecoxib and lyophilized formulations of curcumin-celecoxib loaded NPs, were analyzed in the region of 500-4000cm^{-1} wave number against % transmittance of the functional groups presents in the samples.

2.7 In-vitro drug release study:

2.7.1 In vitro drug release study for nanoparticles:

To ensure release of both the drugs at the PH of skin, invitro drug release studies were conducted in triplicate for both nanoparticles and the final dosage form which was CP gel. Dialysis diffusion bag in bath shaker was used to study in vitro drug release (Jana *et al.*, 2014). Nanoparticle pellet containing 5mg of each drug was suspended in 3ml of phosphate buffer of PH 5.5 and this suspension was placed within dialysis tube which was tied at both ends, incubated in 35ml of phosphate buffer of the same PH. At predetermined time interval of 1, 2,3,4,5,6,7,8,12,24 and 48 hours 1.5 ml of sample was withdrawn from each beaker and 1.5 ml of fresh phosphate buffer was added in order to maintain the sink conditions. The release profile from nanosuspension was continued for 48 hours. Afterwards these

samples withdrawn were analyzed under U.V visible spectrophotometer at wavelengths of 252 and 421, for celecoxib and curcumin respectively.

The calibration curve of both drugs in buffer of PH 5.5 was used to quantify the amount released of both drugs at specific time. Further the percentage of drugs released at specific time and the cumulative percentage of drugs released within 48 hours were calculated. The following formulae were used to calculate the cumulative drug release and amount of drug at specific time (Chandrasekaran *et al.,* 2011).

Concentration of drug (µg/ml) = (slope × absorbance) ± intercept

$$\text{Amount of drug in mg/ml} = \frac{\text{concentration} * \text{dissolution bath volume} * \text{dilution factor}}{1000}$$

$$\text{Cumulative percentage} = \frac{\text{volume of sample withdrawn}}{\text{bath volume}} * Pt + P(t-1)$$

Where Pt = Percentage release at time ‘t’.

Where P (t – 1) = Percentage release previous to‘t’

2.7.2 In vitro drug release study for nanoparticles loaded CP-934 gel:

The release pattern of both the drugs from nanoparticles gel was studied by conducting drug release study from gel using similar method as explained earlier (2.7.1) for drug release study for nanoparticles. The drug release study for gel was also conducted in triplicate. Gel was formulated by suspending a predetermined quantity of nanoparticles in CP solution. The yield of the gel was calculated by weighing the amount of gel produced and amount of both drugs in 1gm of gel was calculated by unitary method. Further release studies were conducted on this gel formulation through method described earlier. The release profile of gel was continued for 48 hours.

2.8 Characterization of nanoparticles loaded CP-934 gel:

The nanoparticles loaded CP gel was characterized on the basis of spreadability, visual inspection, PH and stability at room temperature (Gupta *et al.*, 2010, Pathan *et al.*, 2018, Ubaid *et al.*, 2016, Zeb *et al.*, 2016). Table 2.5 summarizes the desired outcomes as established by literature review for CP gel.

Table 2.5 Parameters studied for Gel

Parameters studied	Ranges of responses	References
Physical parameters	Homogeneity, clarity and presence of clog	(Gupta, Mishra *et al*. 2010)
Viscosity	7000-10,000 cps	(Talele, Nikam *et al.* 2017)
Drug content	70% - 90%	(Zeb, Qureshi *et al.* 2017)
Spreadability	Good Spreadability property	(Pathan, Munde *et al*. 2018)
Stability studies	Stable gels, no presence of clog or change in physical parameters.	(Ubaid, Ilyas *et al.* 2016)

2.8.1 Spreadability of nanoparticles loaded CP-934 gel:

The measure of Spreadability of semisolids is an important parameter in order to study their flow properties. To measure the Spreadability of nanoparticles loaded gels made from different concentrations of CP solution, a modified method to was used to calculate the spread. According to this method about 0.5 grams of gel from each formulation was placed on a clean glass slide marked with a center on 1cm^2 area. Another glass slide was placed on this glass slide for five minutes and gel was allowed to spread (Pathan *et al*., 2018). Afterwards the Spreadability of the gels was calculated as the area of the circle with the help of formula:

$$A = \pi r^2$$

2.8.2 Viscosity:

Brookfield viscometer was used to measure the viscosity of nanoparticles loaded gels. Spindle number 64 was used and it was rotated at 10 rpm and viscosity was recorded**.**

2.8.3 Rheological studies:

Brookfield viscometer was utilized to study the rheological behavior of the nanoparticles loaded gel. To study the effect of shear or stress, viscosity was recorded at different speeds and flow pattern of gels was studied.

2.8.4 PH of gel:

The PH of the gel formulations was calculated with the help of PH meter. The electrode of the PH meter was dipped in the gel after calibration and values for PH were recorded for each formulation in triplicate.

2.8.5 Drug content determination:

To determine the drug content of gel 1gm of gel was dissolved in 100 ml of PBS of PH 5.5 and left to stir for 48 hours. After 48 hours all the formulations were analyzed on UV visible spectrophotometer at wavelength of 252 and 421 nm for celecoxib and curcumin respectively.

2.8.6 Homogeneity and content uniformity:

Visual inspection was carried out to inspect the homogeneity of the nanoparticles loaded gel formulation. The gels were inspected for uniform distribution of drug substance and presence of any aggregates or clumps was recorded.

2.8.7 Clarity:

Clarity of nanoparticles loaded gels was inspected with visual inspection and presence of turbidity was recorded in different concentrations of CP gel.

2.9 Ex vivo permeation test:

2.9.1 Preparation of skin:

Excised mouse skin of swiss albino mice was used to study ex-vivo permeation of the gels (Jana *et al.,* 2014). For this purpose, skin was first prepared, anesthetic ether was used to sacrifice the animals and full thickness abdominal skin was obtained. Skin hair were removed using animal hair clipper, abdominal fat and adhering tissues were removed with the help of scalpel. The obtained skin was washed with PBS and stored in the freezer for later use.

2.9.2 Franz diffusion cell apparatus:

The most common type of static diffusion cells used for permeation studies is Franz Diffusion Cell apparatus. This assembly consisted of vertical diffusion cells, magnetic stirring control system, and heating circulation system to study ex vivo permeation with a programmable temperature control device. The effective permeation area of the cells was 0.77cm^2. Each cell consisted of a donor and receptor compartment. The receptor compartment had the capacity of 5ml PBS 7.4 which was filled in it and rotations were set to 500 rpm. The temperature of receptor compartment was maintained at 37° C ± 0.5° C. Mouse skin was mounted between the donor and receptor compartment with stratum corneum on the upper side and epidermis on the lower side. The dermal side of the skin

was in contact with the receptor medium. The complete assembly was securely clamped after application of 1.5 gram of gel on the skin loaded in the donor compartment. 1ml of sample was withdrawn through the sampling port at predetermined time intervals of 0.5 hour, 1 hour, 2 hours, 4 hours, 8 hours, 12 hours, 16 hours, 20 hours, and 24 hours. After each sample withdrawn the receptor phase was immediately replenished with equal volume of fresh buffer to maintain the sink conditions. The collected samples were analyzed for both the drugs on UV visible spectrophotometer after making suitable dilution with PBS 7.4 (Zeb *et al.*, 2016).

The following formulae were used to calculate cumulative permeation of both drugs for 24 hours.

$$Qn = \frac{Cn*Vr+\sum Ci*Vs}{A}$$

Where;

- Qn is the cumulative amount of drug permeated per unit area (µg/cm^2) corresponding to the time of nth sample,
- Cn is the drug concentration in receptor fluid at the time of nth sample
- ∑Ci is the sum of drug concentration in receptor fluid previous to the time of nth sample (at the time of ith (n−1) sample)
- Vr is the volume of receptor solution (5 mL)
- Vs is the volume of sample withdrawn (0.5 mL)
- A is the effective permeation area of diffusion cell (0.77 cm2).

The cumulative amount of curcumin and celecoxib permeated per unit area (Qn) was plotted with respect to time and steady-state flux Jss expressed as µg/cm2/h for 24 hours was calculated. Permeability coefficient (Kp) was calculated by dividing the steady-state flux (Jss) by the initial amount of drugs in donor compartment.

2.10 Application of kinetic models to study drug release:

The models for drug release are used in order to study the release pattern of drugs. These models explain how the drug behaves in the release medium. This helps in extrapolating and predicting the *in-vivo* release of the drugs ,through in-vitro evaluation of drug release pattern.

There are a number of kinetic models for drug release that have been described over the years

Zero order kinetics:

This kinetic model is used to explain constant release of drug from the formulation. To check drug release kinetics according to zero order kinetics, a graph was plotted between "cumulative drug release and time" .Slope of this graph represented zero order release constant. Zero order release was represented by following equation:

$$W = k_1 t$$

Where;

W= cumulative drug release

t= time in hours

k_1= zero order release constant

First order kinetics:

This kinetic models is applied in situations where sink conditions are maintained properly. A graph was plotted between log of cumulative drug release versus time and represented by following equation.

$$\ln(100-W) = \ln 100 - k_2 t$$

Where;

k_2= first order release constant

Hixon Crowel's model:

This model explains drug release by more than one mechanism such as dissolution, and erosion followed by diffusion. The following equation describes hixon crowel's model.

$$(100)^{1/3} - 100^{1/3} - W = -k_3 t$$

Where k_3= Hixon release constant

Higuchi Diffusion model:

Higuchi model explains drug release from matrix based on diffusion in a non-erodible way. To study this model average cumulative drug release was plotted against square root of time. This model is explained by the equation given below.

$$W = k_4 t$$

Where k_4= Higuchi dissolution rate constant

Korsmeyer Peppas diffusion/relaxation model:

This model is used to describe drug release of in a sustained manner. Ficks laws of diffusion are used to describe drug release in korsmeyer peppas model. This model is explained by the following equation:describes drug release versus elapsed time with an exponential function and explained by following equation:

$$Mt/M\infty = k_5 t^n$$

Where $Mt/M\infty$ = fraction of drug release at time t

k_5= constant incorporates structural and geometric characteristics of drug release device

^{n}= diffusion exponent for drug release

In this model value of n describes mechanism of drug release. n= 0.45 describes Fickian diffusion and $0.45 < n > 0.9$ illustrates non-Fickian diffusion n = 0.89 describes case II or zero order release while n > 0.89 explains super case II release.

2.11 Stability studies of gel formulation:

Stability studies of nanoparticles loaded CP gel were performed at room temperature for six months. Accelerated stability testing was also carried out at 40C and 75% humidity in stability chamber. Physical parameters such as PH, homogeneity, phase separation were observed and recorded (Ubaid *et al.,* 2016).

2.12 Statistical analysis:

All experiments were carried out in triplicate and presented as an average of triplicate samples with standard deviation. One-way ANOVA was utilized in order to evaluate the difference among the groups with level of significance < 0.05 considered statistically significant.

2.13 *In-vivo* experiments:

The acute model of Carrageenan induced inflammation was used to the study the effect of nanoparticles loaded gels in inflamed hind paw of mice. A slightly modified method from those previously reported was utilized in this regard (Rasheed *et al.*, 2018).

2.13.1 Animals:

Male albino mice having age of 4–5 weeks and weight of 25–30g were selected for in vivo experiments. All animal activities were carried out in pathogen free zone of Quaid-i-Azam University, Islamabad, following procedures outlined in guideline for the care and use of laboratory animal Quaid-i-Azam University under the approval of Bio-Ethical Committee Code (BEC-FBS-QAU2019-130). Stainless steel cages were used to house the animals in conditions of Animals were housed in 23 ± 0.5°C and humidity of 10% in 12h light-dark cycle. The acclimatizing period for animals was one week before the study when they were fed with standard diet and water. All animal activities were performed following procedures of "Principles of Lab Animal Care" from NIH publications for investigating pain in living animals. All the experimental animals were used once and all the activities were performed from 8:00am to 6:00pm. There were three mice in each group and four groups were formed. One was normal group. There was negative control treated only with carrageenan. There were two positive control groups one was treated with a hydroalcoholic CP gel of curcumin and celecoxib plain drug combination while one was treated with curcumin-celecoxib NPs loaded CP gel. The right hind paw of three groups of mice was injected with carrageenan (100ul/paw). The gels were applied after 45 minutes of injecting carrageenan into the hind paw of mice. The dose calculated for both drugs was approximately 8mg/kg of body weight. This dose was incorporated as curcumin-celecoxib plain gel as well as curcumin-celecoxib NPs loaded gels. All the groups normal group were subjected to intraplantar injection of carrageenan and gels were administered topically. Various behavioral experiments were conducted on all the groups of animals.

2.14 Behavioral experiments:

2.14.1 Mechanical allodynia:

Mechanical allodynia was assessed using Von Frey filaments (Stoelting, USA) according to method described previously (Ullah *et al.*, 2018). Mice were placed in clear plastic chamber on a stainless-steel mesh floor and allowed to acclimatize. Von Frey filament was

applied on the plantar surface of the right hind paw with gradual increase in the filament size. The filament size was directly proportional to the amount of applied force and was measured in grams. This test was conducted for all the four groups and the trend in pain threshold level was recorded. The strength of the Vonfrey filaments were taken as the level of pain tolerance in mice. The readings were taken at 0 hour, 2 hours, 4 hours and 6 hours for all groups. The second reading for positive control groups were taken after 1.5 hour of applying gels.

2.14.2 Thermal hyperalgesia:

Hot plate test was conducted in order to evaluate thermal hyperalgesia in mice. For this purpose, the temperature of hotplate was adjusted to 55–60°C. Animal were placed on hot plate one by one and, paw withdrawal or paw licking response was observed. The time taken to evoke such reflexes was noted and designated as paw withdrawal latency. The Cut off time for heat stimulation was 40 seconds (Ullah *et al.*, 2018).

2.14.3 Paw edema measurement:

Investigation of paw edema in mice was evaluated with the help of Dial Thickness Gauge meter (No. 2046 F, Mitutoyo, Kawasaki, Japan). The edema in the hind paw was measured after induction of carrageenan in negative control group of animals. For positive control groups the paw edema was measured after 1.5 hours of application of gels. The time interval at which paw edema in all groups was measured was 2hours, 4 hours and 6 hours.

CHAPTER 3

RESULTS

3. RESULTS

3.1 Preparation of Standard Calibration Curves

3.1.1 Standard Calibration Curve of Curcumin and Celecoxib in Methanol:

After formation of specific dilution (table 2.1), the absorbances for both drugs were checked against each dilution (table 3.1). These absorbance values were recorded and a graph was plotted to obtain the equation of calibration curve (figure 3.1).

Table 3.1 Calibration Curve in Methanol

Concentration ug/ml	Absorbance Celecoxib	Absorbance Curcumin
0.2	0.165	0.195
0.4	0.338	0.384
0.6	0.442	0.557
0.8	0.6	0.695
1	0.691	0.829
1.2	0.862	0.995

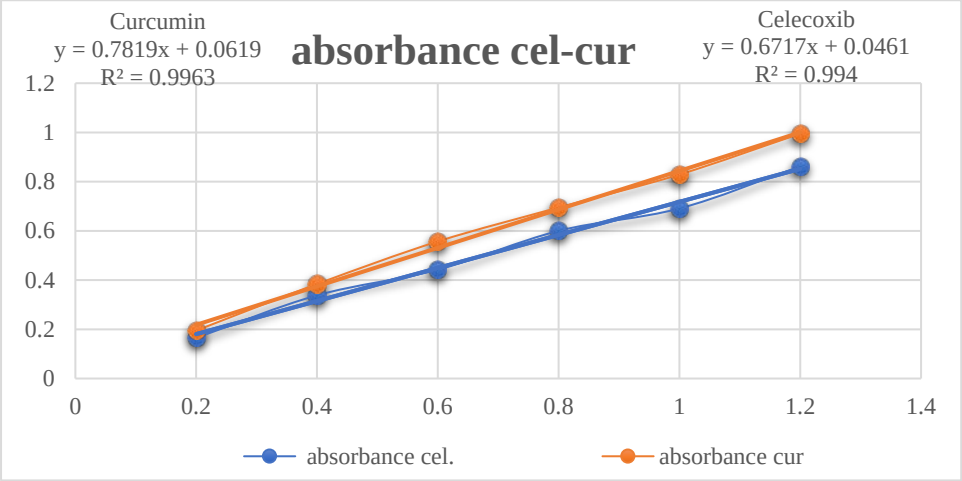


Figure 3.1 Calibration Curve in Methanol

3.1.2 Standard calibration curve in phosphate buffer PH 5.5:

Standard calibration curve in phosphate buffer of 5.5 PH was obtained after making suitable dilutions (2.3.1.2.2) and checking their absorbances (table 3.2).

Table 3.2 Calibration Curve at 5.5 PH

Concentration	Absorbance Curcumin	Absorbance Celecoxib
0.6	0.01	0.154
0.8	0.034	0.202
1	0.07	0.249
1.2	0.093	0.31
1.4	0.12	0.376
1.6	0.154	0.429
1.8	0.183	0.5
2	0.19	0.533

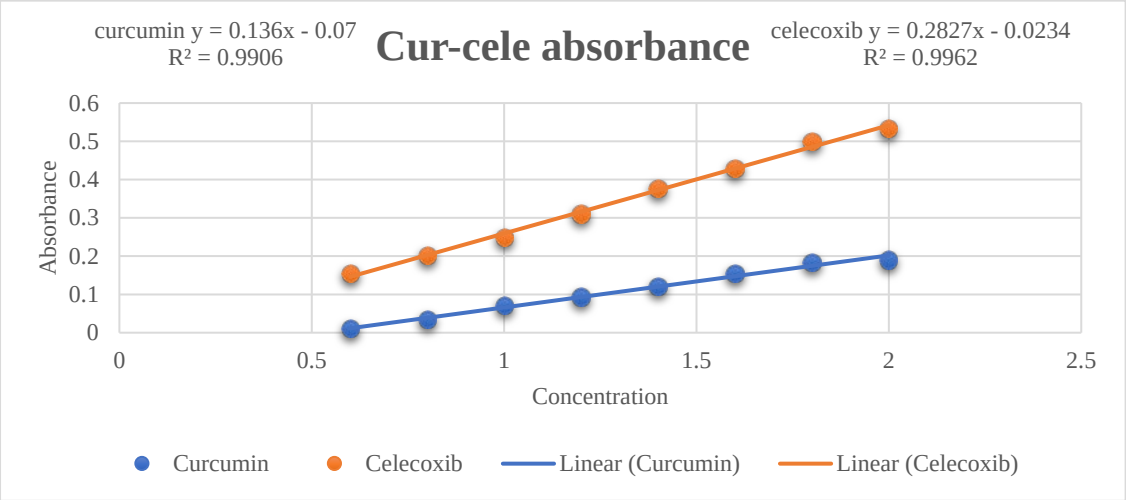


Figure 3.2 Calibration Curve at 5.5 PH

3.1.3 Standard curve in phosphate buffer PH 7.4:

After making phosphate buffer of PH 7.4, suitable dilutions were made (2.3.1.2.3) and absorbances were checked on UV vis spectrophotometre (table3.3).

Table 3.3 values of absorbance for various concentrations

Concentration	Absorbance Curcumin	Absorbance Celecoxib
0.6	0.464	1.088
0.8	0.53	1.154
1	0.598	1.179
1.2	0.614	1.268

1.4	0.684	1.297
1.6	0.717	1.373
1.8	0.736	1.382
2	0.803	1.466

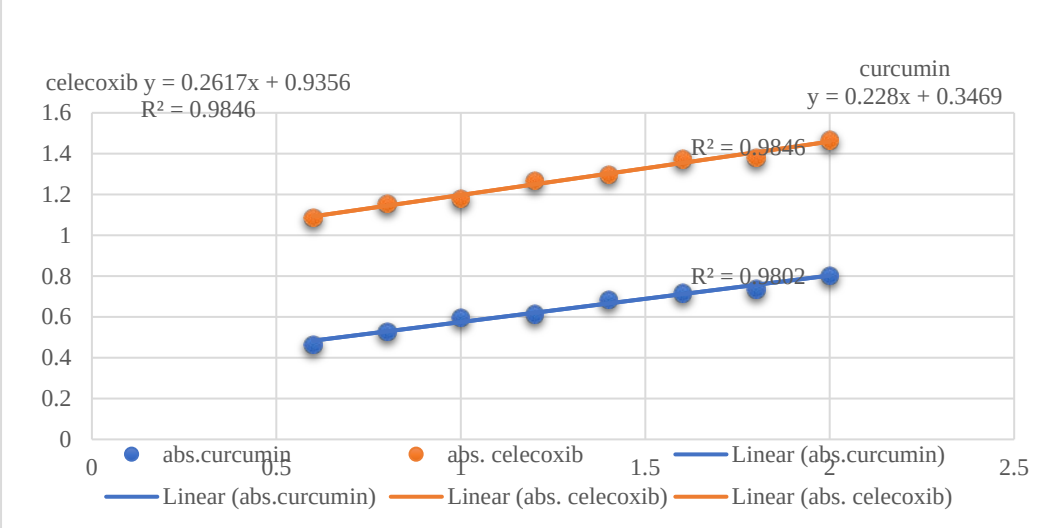


Figure 3.3 calibration curve in PBS 7.4

3.2 Preparation of Nano formulation:

3.2.1 Preparation of blank nanoparticles:

Blank PLGA nanoparticles were prepared by o/w emulsion solvent evaporation method (2.4.1). Prepared emulsions were turbid white in color. The zeta potential was up to -20, the size ranged from 150 nm to 170nm. Table 3.4 summarizes some charecteristics of blank PLGA nanoparticles.

Table 3.4 Characteristics of Blank PLGA Nanoparticles

Form.	E:A to PVA ratio	Drug content	% of PVA soln.	Physical appearance	Size nm	Suspend ability
A.1	1:1	Blank	2%	White turbid	170 ± 5	Easily suspended
A.2	1:2	Blank	2%	White turbid	120 ± 3	Easily suspended
A.3	1:3	Blank	2%	White turbid	90 ± 5	Easily suspended

3.2.2 Preparation of drug loaded nanoparticles:

PLGA nanoparticle formulation containing both the drugs was prepared by o/w emulsion solvent evaporation method (2.4.2). Various stirring speeds were studied as a parameter which effects the size of Nano particles.

The decrease in concentration of PVA solution resulted as increase in size and also increase in EE (Encapsulation Efficiency). The increase in drug concentration resulted as increase in size and EE.

The size considered optimal for topical drug delivery for musculoskeletal disorders is between 20nm to 200nm. Therefore, the optimization was targeted at attaining size within this range with maximum drug entrapment. Figure 3.4 illustrates the visual characteristics of drug loaded nanoparticle suspension.

Figure 3.4 nanoparticle suspension

Table 3.5 summarizes some parameters which were studied and adjusted along with the respective results which were obtained.

Table 3.5 parametres studied and adjusted

Form.	EA:PVA	Amount of each Drug	PVA %	Appearance	Size	Entrapment efficiency	
						Celecoxib	Curcumin
B1	1:1	5mg	2%	Pale Yellow turbid emulsion	185 ± 5	70%±5	80%±4
B2	1:1	7.5mg	2%	Yellow turbid emulsion	350nm	75%±2	88%±2
B3	1:1	10mg	2%	Dense yellow emulsion	450nm	89%±5	90%±4
C1	1:1	7.5mg	1%	Yellow turbid emulsion	589.9nm	90%±3	98%±1
C2	1:1	7.5mg	1.5%	Yellow turbid emulsion	533.3nm	85%±3	94%±2

3.2.3 Particle Size and Zeta Potential Analysis Protocol

The particle size, distribution and zeta potential of the samples were determined using the Zeta sizer Nano ZS 90 (Malvern Instruments; Worcestershire, UK), equipped with software (version 6.34) and a He-Ne laser at a wavelength of 635 nm and static scattering angle of 90 degree. Briefly 10 µl of the sample was mixed with 1 ml of deionized water and vortexed for 2 minutes followed by analysis with zeta sizer. Each result displayed was measured in triplicate (table 3.6, table 3.7)

Table 3.6 Size Distribution Report by Zeta Sizer

			Size (d.nm):	**% Intensity:**	**St Dev (d.n...**
Z-Average (d.nm):	177.2	Peak 1:	187.0	100.0	44.74
PdI:	0.040	Peak 2:	0.000	0.0	0.000
Intercept:	0.966	Peak 3:	0.000	0.0	0.000
Result quality:			**Good**		

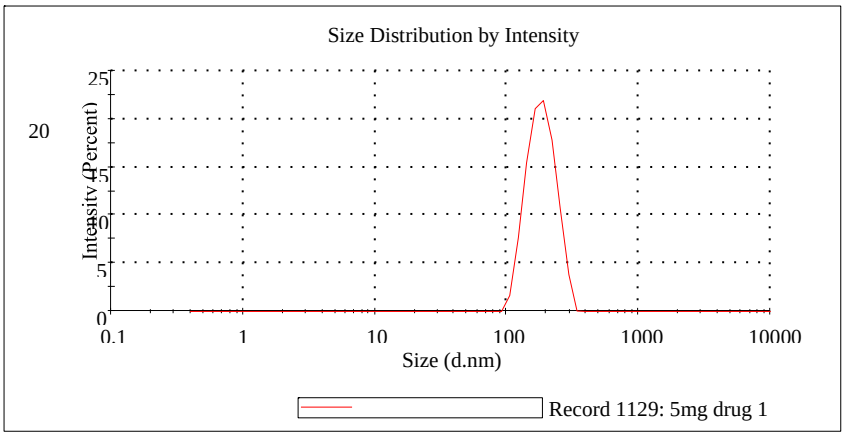


Figure 3.5 Size Distribution Report by Zeta Sizer

Table 3.7 Size Distribution Report by Zeta Sizer

			Size (d.nm):	**% Intensity:**	**St Dev (d.n...**
Z-Average (d.nm):	185.8	Peak 1:	208.2	100.0	73.52
PdI:	0.092	Peak 2:	0.000	0.0	0.000
Intercept:	0.938	Peak 3:	0.000	0.0	0.000
Result quality:			**Good**		

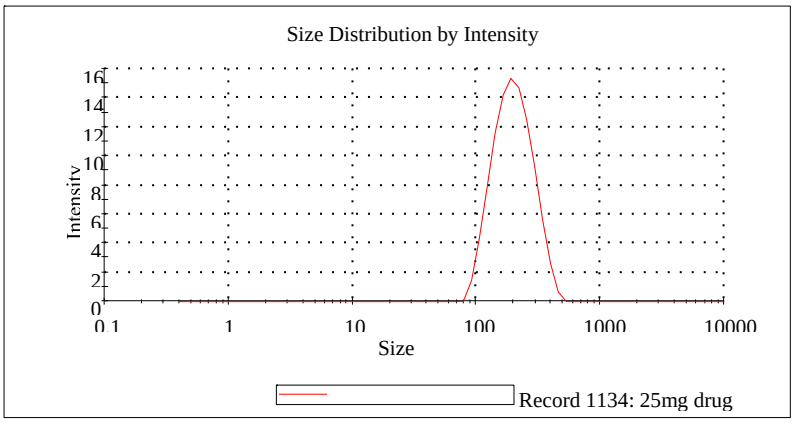


Figure 3.6 Size Distribution Report by Zeta Sizer

3.2.4 Effect of PVA concentration on particle size:

The decrease in concentration of PVA solution resulted as an increase in size of Nanoparticles and also increase in Encapsulation efficiency (figure 3.7).

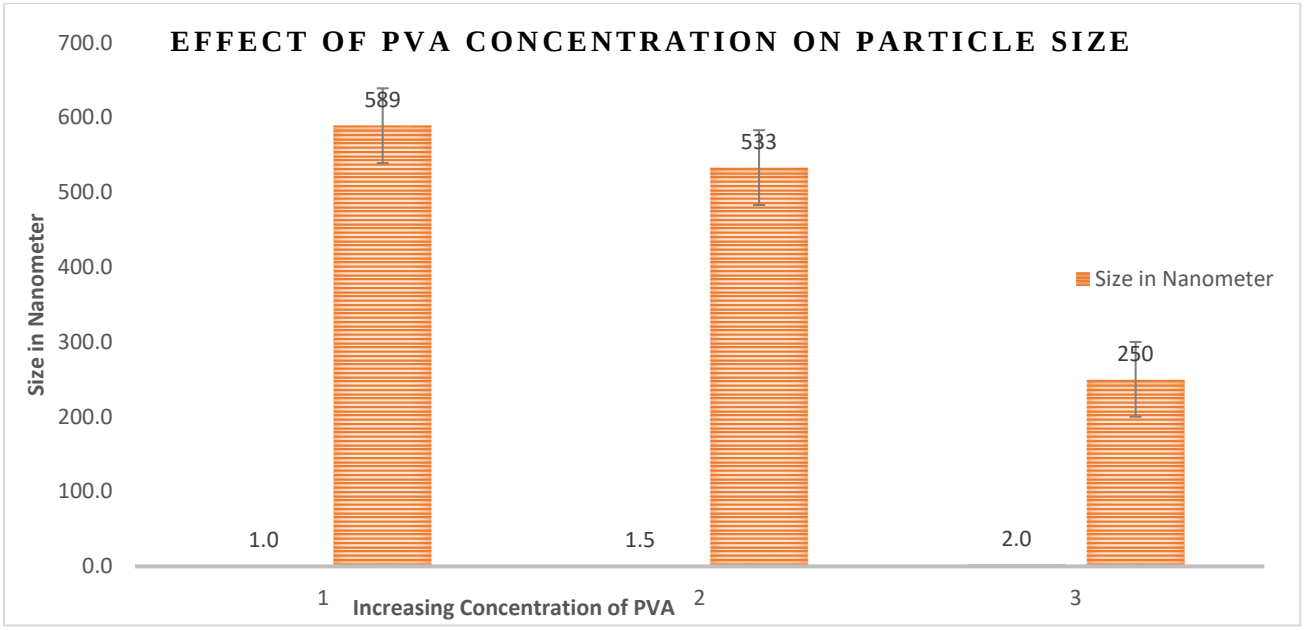


Figure 3.7 Concentration of PVA and particle size in nanometer

3.2.5 Effect of drug loaded on size and E.E:

Different concentrations of both drugs were loaded into nanoparticles and the effect was observed included 5 mg. of each drug, 7.5 mg. of each drug and 10 mg. of each drug. It was observed that as the drug load was increased, the size of nanoparticles also increased and consequently so did the E.E.

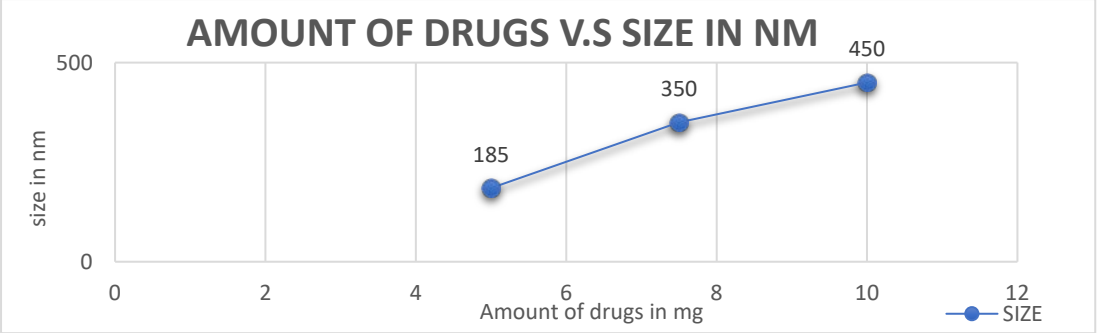


Figure 3.8 Effect of Amount of Drug on Particle Size

3.2.6 Zeta potential:

The zeta potential is a measure of repulsion within the nanoparticle suspension due to presence of alike charges. Higher value of zeta potential corresponds to less aggregation and increased stability of Nano formulation. The values of zeta potential for all the Nano formulations formulated during the course of this study were found to be -18 ± 1.83 . Although these values signify some aggregation in the nanoparticle system but the nanosuspensions were freeze dried for later use and hence presence of lesser charge did not pose any problem.

3.2.7 Polydispersity Index:

PDI is a measure of level of dispersibility within the nanoparticles suspension. The values of PDI were found to be within 0.01 and up to 0.9.the smaller value of PDI corresponds with good dispersibility of nanoparticles in the system (table 3.8).

Table 3.8 Polydispersity Index

Drug Content	PDI			Mean ± SD
	Number of Observations			
	1	2	3	
5 mg each drug	0.268	0.128	0.124	0.18 ± 0.07
7.5 mg each drug	0.494	0.587	0.467	0.54 ± 0.06
10 mg each drug	0.833	0.815	0.823	0.824 ± 0.01

3.2.8 Surface morphology:

Scanning electron microscope was use to visualize nanoparticles and study their morphology (2.6.3). The images which were obtained with the help of SEM are depicted in figure 3.9.

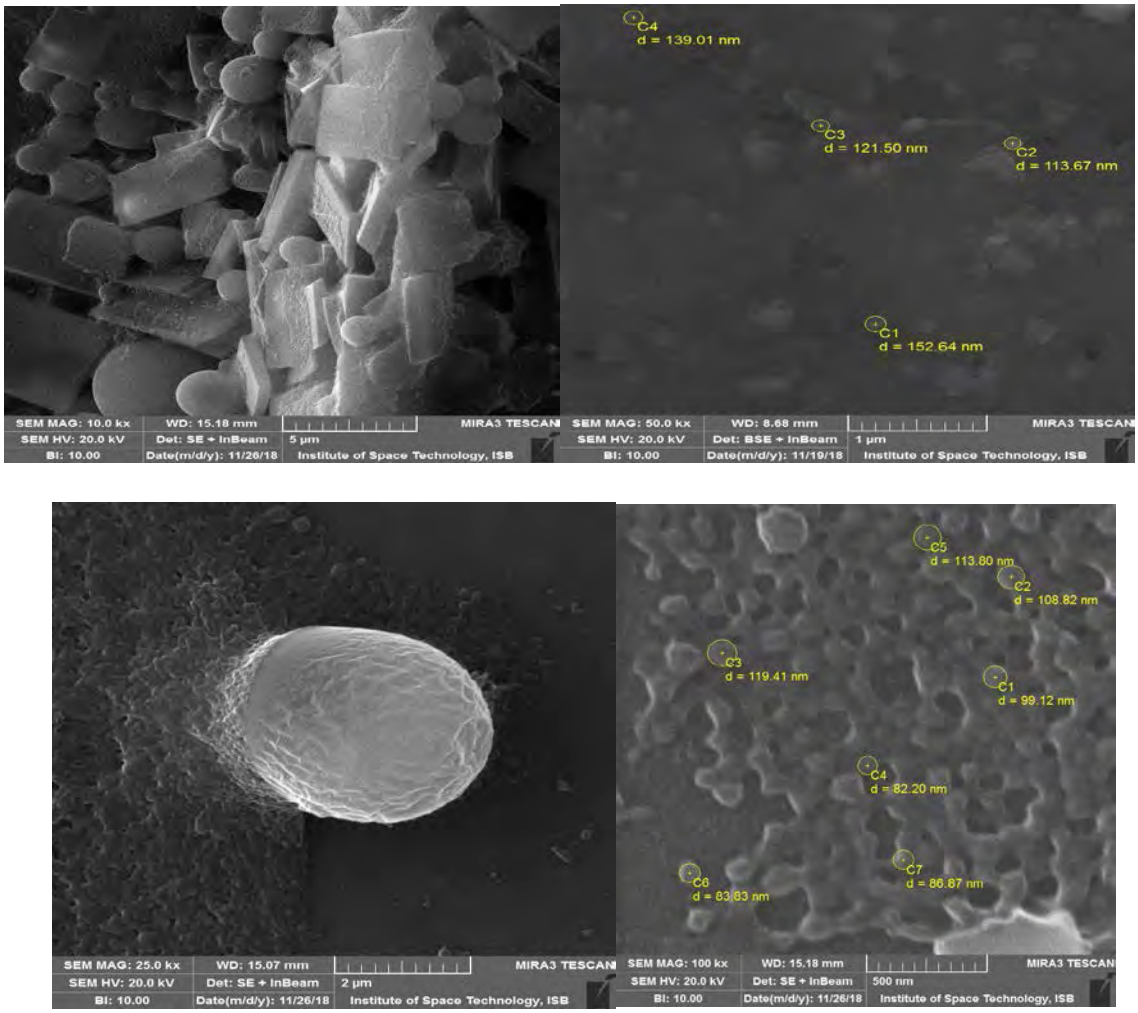


Figure 3.9 SEM images of nanoparticles

Spherical nanoparticles can be seen in the SEM images. The size range was found to be between 100nm to 200nm. Some free drug crystals can be also be seen in the images, adhered to nanoparticle surfaces.

3.2.9 FTIR Analysis:

FTIR analysis was carried out in order to study the chemical nature of formulation (2.6.7). FTIR of PLGA the polymer, both the drugs separately and drug loaded nanoparticles was carried out (figure 3.10). Various fuctional groups which were detected were identified with the help of their characteristic wavelength through FTIR analysis.

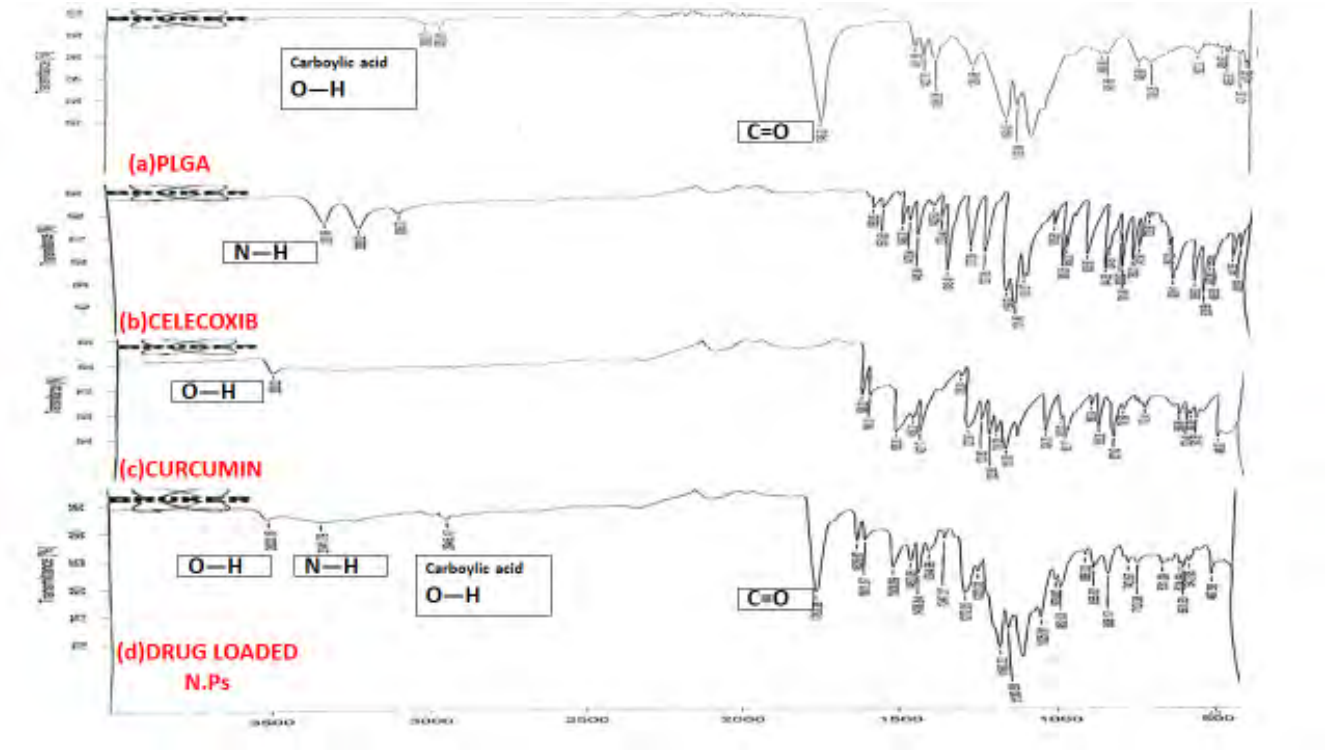


Figure 3.10 FTIR of polymer, drugs and Nano formulation

3.2.9.1 Interpretation of FTIR:

Table 3.9 summarizes the various functional groups which were identified in FTIR analysis. It was observed that there was no addition of chemical bonds in prepared Nano formulation. Moreover, all of the functional groups identified in the FTIR spectra of polymer and drugs can be identified in that of nano formulation, this indicated the compatibility between polymer and drugs in final formulation.

Table 3.9 Interpretation of FTIR

Functional Group	Characteristic Absorbance Range	Celecoxib	Curcumin	PLGA	Nanoparticles
N—H Stretch	3300 —3500	3331.98, 3226.02	NIL	NIL	3341.79
C—H	3000—3100	3096	NIL	NIL	NIL
N—H Bending	1550—1640	1561.68, 1593.05	1601.01	NIL	1601
O—H STRETCH	3200 —3600	NIL	3508	NIL	3503.18

C=O STRETCH	1640—1690	NIL	1626.22	NIL	1626
C≡C	1400 —1600	NIL	1427.17,1455.37,1505.30	1422,1451	1426,1508
Carboxylic acid 0-H	- 3300	NIL	NIL	2995.5,2950	2945.17
Ester C=O	1735 -1750	NIL	NIL	1745.52	1753.38

3.3 Preparation of nanoparticles loaded CP-934 gels:

Nanoparticles loaded CP gels were prepared with the method previously described in methodology (2.5). Different concentrations of CP were made with water and gel from each concentration was formulated. Afterwards all the nano-gel formulations were characterized on the basis of PH, Spreadability and visual inspection. Figure 3.11 depicts the visual representation of nanoaprticles loaded CP gels.

Figure 3.11 Visual Representation of CP

Table 3.10 summarizes the formulation of nanoparticles loaded CP gel along with results of visual inspection.

Table 3.10 Various Concentrations of CP Gel

Concentration of CP	No. of drops TEA	Results on visual inspection
0.5%	3	Uniform homogenous, good clarity
1%	2	Uniform homogenous gel, good clarity
1.5%	2	Uniform homogenous gel,excellent clarity,no lumps Excellent clarity, no visual lumps
2%	1	Unclear turbid, presence of lumps
2.5%	1	Air bubbles present, unclear turbid, lumps present

3.3.1 PH of gel formulations:

The PH of all gel formulations was found to be between 5 and 7 ,which is the desired PH range for topical formulations (Talele *et al.*, 2017).

3.3.2 Spreadability:

The Spreadability values of all the formulated gels with different concentrations of CP were measure through glass slide method. The spreadability was calculated as the area of the circle in mm^2.

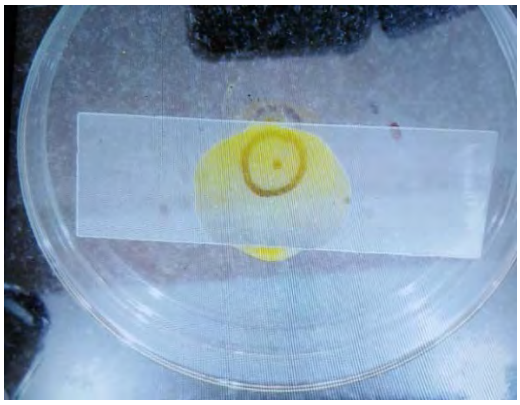


Figure 3.12 Measurement of Spreadability of Gel

Table 3.11 summarizes the values of Spreadability for nano gel formulated with each concentration of CP:

Table 3.11 Spreadability Values

Concentration of CP	Spreadability in mm^2
0.5%	1017
1%	379.94
1.5%	314
2%	226.86
2.5%	132.66

It was noted that the increase in CP concentration lead to a decrease in spreadability and consequently an increase in viscosity (figure 3.13). The Nano gel with the CP concentration of 1.5% was chosen as optimized formulation due to its better clarity, optimum Spreadability and PH between 5.2 to 5.5 which most closely resembles the PH of the skin which is 5.5.

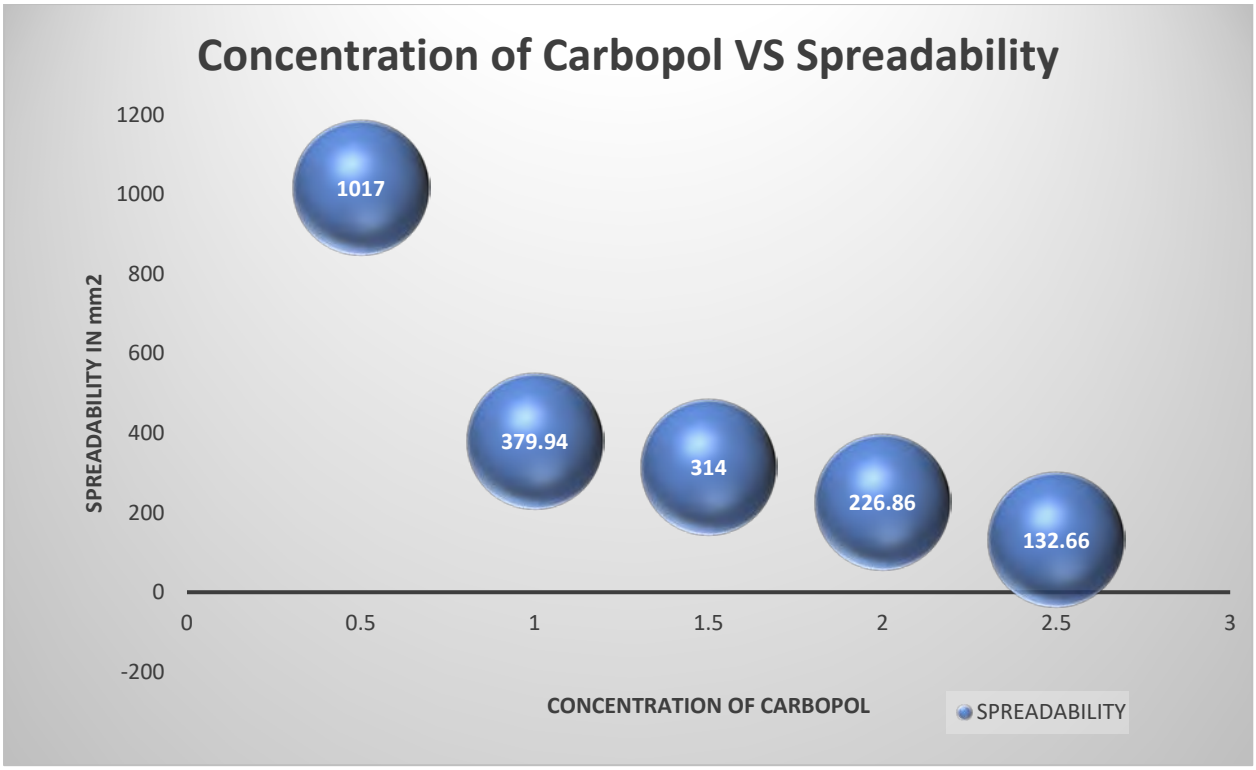


Figure 3.13 Concentration of CP VS Spreadability

3.3.3 Viscosity:

Brookefield viscometer was used to measure the viscosity of gels (2.8.2). The viscosity of the optimized gel formulation was found to be 9728 centipoise.

3.3.4 Rheological behavior:

The spindle number 64 of Brookefield viscometer was used and the effect of shear stress was studied on the gel (Zeb *et al*., 2016). The results indicated that the gels followed a non-Newtonian flow (figure 3.14). As the speed of the rotations of viscometer increased, viscosity was found to decrease. When the stress was removed the viscosity was regained by the gel. This effect is termed as shear thinning and it is a desirable property for semi solids.

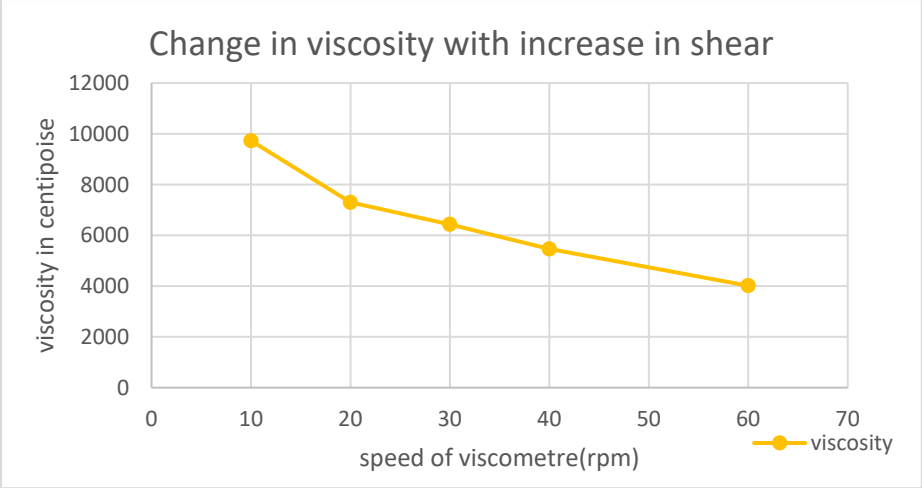


Figure 3.14 Change in viscosity with increase in shear

3.4 Stability of gels:

The optimized gel formulation was studied for stability in the accelerated stability chamber as well as stability at room temperature was also studied (2.11). Table 3.12 depicts the results of accelerated stability testing at temperature 40C and 75% humidity level.

Table 3.12 Results for accelerated stability testing

Time in months	Ph of gel	Physical parameters
After 1 month	5.2 to 5.1	Homogenous, clear
After 2 months	4.5 to 4.7	Homogenous, less viscous
After 3 months	4.3 to 4.7	Lumps formation, phase separation

Accelerated stability testing revealed instability in CP gels. The excessive heat and humidity has the effect of decrease in PH and the crosslinking among CP and TEA seems to be disrupted. This results in loss of gel structure and decrease in PH. However the thixotropic property of CP was responsible for such behavior of the gels. As the temperature conditions were reversed the gels regained their original structure .

Table 3.13 represents the results for stability testing at room temperature.

Table 3.13 Results for stability testing at room temperature

Time in months	Ph of gel	Physical parameters
After 1 month	5.0 to 5.1	Gel structure intact
After 2 months	5.2 to 5.1	Gel structure intact
After 3 months	4.9 o 5.1	Gel structure intact

The CP gels exhibited good stability at room temperature. The gel structure was retained and the PH, homogeneity clarity were all found to be within the range at room temperature.

3.5 Drug content determination:

Drug content measurement was conducted in triplicate in order to find out homogeneity of drug content (2.8.5). After stirring gels with PBS for 48 hours dilutions were made in order to measure absorbance through UV spectrophotometer. The following formula was used to calculate drug content in gels.

$$\frac{drug\ content\ observed}{drug\ content\ loaded} * 100$$

Table 3.14 summarizes the drug content in gel formulations:

Table 3.14 Drug Content

Formulation	Drug content celecoxib Mean ± S. D	Drug content curcumin Mean ± S. D
D1	89% ± 5	85% ± 3
D2	84% ± 5	85% ± 5
D3	78% ± 3	80% ± 2

3.6 Permeability test:

Franz diffusion cell apparatus was used to study permeability of drugs from gel across the skin.

Figure 3.15 Franz Diffusion Cell apparatus

The samples were withdrawn upto 24 hours. The cumulative permeability was calculated after quantifying the amount of both drugs with UV spectrophotometer.Table 3.15 summarizes the cumulative permeability values for curcumin and celecoxib. The steady state flux JSS was also calculated for both drugs and so was the permeability coefficient.

Table 3.15 Permeability Values

Drugs	Cumulative Permeability	JSS	Kp
Curcumin	148.35	6.18	0.012
Celecoxib	137.21	5.71	0.01

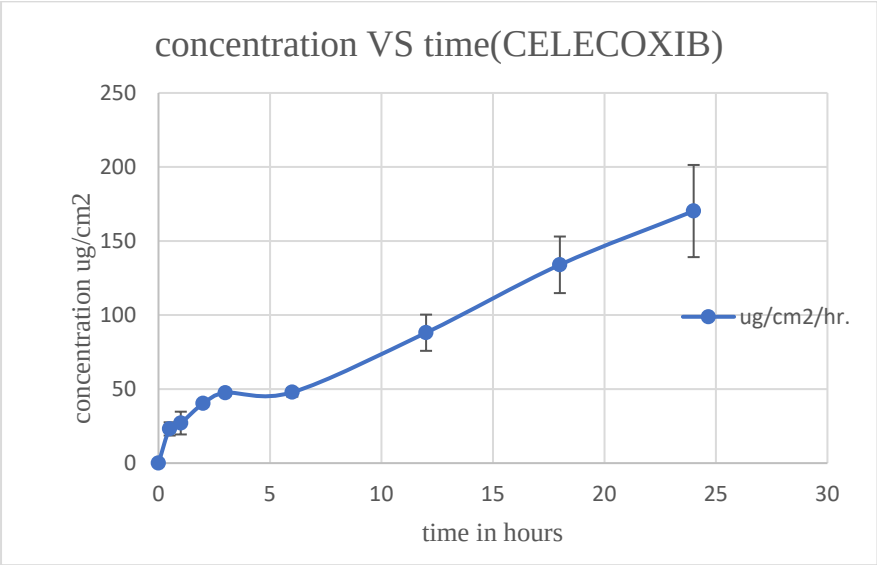


Figure 3.16 Permeability values for Celecoxib

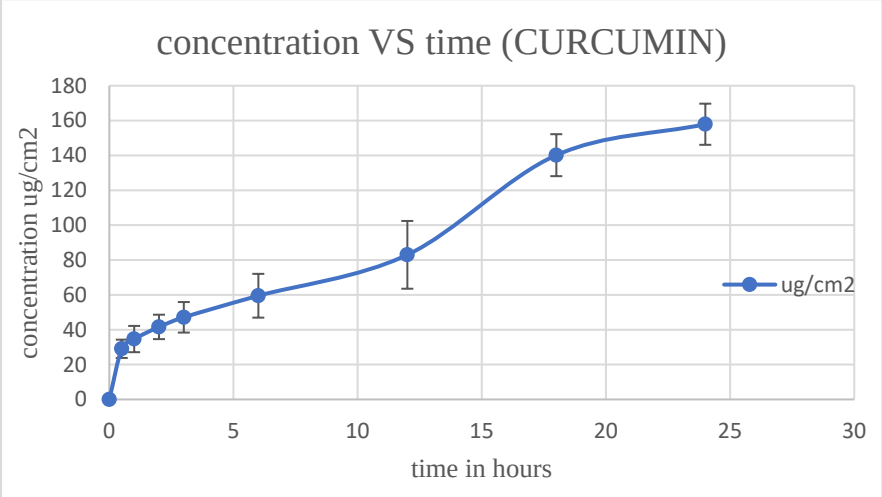


Figure 3.17 Permeability values for Curcumin

3.7 Drug release profile:

3.7.1 Drug release profile for nanoparticles:

Dialysis diffusion bags were utilized in the bath shaker to study drug release from Nano particles at PH 5.5. The release profiling was continued for 48 hours for Nano particles. The mean cumulative drug release for each drug was then calculated and plotted on excel against time. These values were added into DD Solver software for the purpose of drug release kinetic models to be applied. The following were the average cumulative release values for celecoxib and curcumin Nano particles.

Table 3.16 Average Cumulative Drug Release from Nano particles

Time in Hours	Average Cumulative Drug Release ± S.D.	
	Curcumin Nanoparticles	Celecoxib Nanoparticles
0	0	0
1	33.49 ± 4.74	35.96 ± 2.40
2	36.49 ± 4.06	39.04 ± 3.62
3	40.14 ± 2.85	42.86 ± 3.79
4	45.25 ± 3.02	46.78 ± 3.94
5	49.80 ± 2.12	50.67 ± 4.16
6	55.05 ± 3.61	54.53 ± 4.21
7	58.70 ± 4.61	58.44 ± 4.20
8	61.86 ± 3.13	62.55 ± 4.46
24	65.85 ± 1.79	66.76 ± 4.89
48	75.77 ± 2.45	70.92 ± 5.46

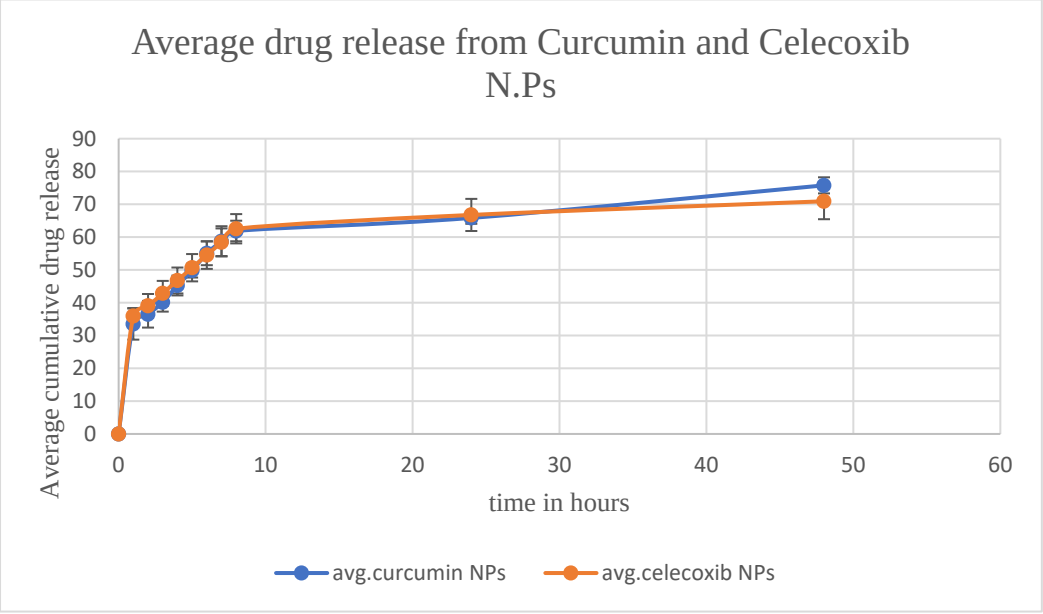


Figure 3.18 Average cumulative drug release of curcumin and Celecoxib Nano particles

The results of cumulative drug release indicate a burst release of both drugs after one hour which can be attributed to some of the free drug molecules still latched to the Nano particles. However, it can be observed that with the passage of time small quantities of both drugs are released over a span of 48 hours. This implies sustained release of drugs from Nano particles. Another observation is that after the reading of 24 hours, there is a marked increase in release of drugs from Nano particles, which resulted in approximately 75.77 ± 2.45 % of Curcumin released. The maximum value of Celecoxib is 70.92 ± 5.46 % released from Nano particles within 48 hours.

3.7.2 Drug release from Nanoparticle loaded gels:

Drug release profiling from gels were carried out in the same method as prescribed in (3.7.1) samples were taken at predetermined time intervals and profiling was carried out for 48 hours. The following table summarizes the average cumulative drug release of both drugs from nanoparticles loaded gels in 48 hours.

Table 3.17 Average cumulative drug release curcumin and celecoxib from N.Ps loaded CP gel

Time in Hours	Average Cumulative Drug Release ± S.D.	
	Curcumin Gel	Celecoxib Gel
0	0	0
1	26.37 ± 2.48	27.36 ± 3.04
2	28.76 ± 2.48	29.25 ± 3.14
3	32.05 ± 2.27	31.41 ± 3.25
4	35.38 ± 2.05	33.59 ± 3.42
5	38.64 ± 1.92	35.80 ± 3.62
6	41.86 ± 1.86	38.04 ± 3.84
7	45.08 ± 1.84	40.27 ± 4.01
8	48.32 ± 1.83	42.41 ± 4.09
24	51.75 ± 1.87	44.59 ± 4.04
48	55.3 ± 1.35	49.98 ± 3.32

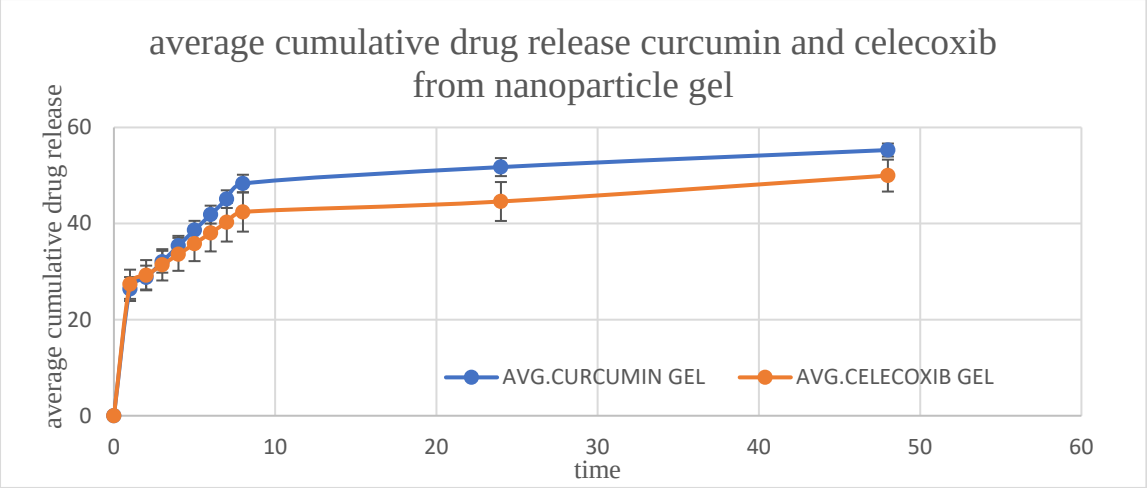


Figure 3.19 Average cumulative drug release of Celecoxib from Gel

3.7.3 Application of ANOVA on drug release patterns:

In order to analyse statistically significant differences among drug release profile of drugs from nanoparticles and from NPs loaded gels, one way ANOVA test was applied on the data. A p value > 0.05 was obtained which indicated no significant difference in the release pattern of curcumin and celecoxib from NPs and from NPs loaded gels.

3.8 Application of Kinetic models to study drug release:

DD solver was used to apply various kinetic models to describe drug release from nanosuspension as well as gels. Various kinetic models were applied and the drug release model with the highest value of regression was considered the best fit model to explain the release pattern of drugs.

Table 3.18 summarizes the values of regression for NPs of Celecoxib and table 3.19 for NPs of Curcumin and signify the best fit model to study drug release from NPs.

Tables 3.18 Kinetic modeling for Celecoxib Nano particles

Higuchi	**Zero order**	**First order**	**K-peppas**	**Hixon crowel**
0.47	-3.58	0.513	**0.978, n= 0.154**	0.560

Table 3.19 Kinetic modeling for Curcumin Nano particles

Higuchi	Zero order	First order	K-peppas	Hixon crowel
0.237	-2.02	0.478	**0.9869,n=0.344**	0.083

It can be observed that Korsmeyer peppas has the highest value of regression in the case of NPs. The value of "n" for both drugs is less than 0.5 which indicates Fickian diffusion of drugs from Nano particles. This implies that the drug release from the polymeric matrix is through classical diffusion which is controlled by thermodynamic forces such as gradient of chemical potential or differences of concentration.

Table 3.20 summarizes the values of regression for various models applied on release of celecoxib from NPs loaded CP gel and table 3.21 summarizes the same for Curcumin.

Table 3.20 Kinetic modeling for Celecoxib from NPs loaded Gel

Higuchi	Zero order	First order	K-peppas	Hixon crowel
-0.16	-2.96	-1.48	0.9885,N=0.156	0.08

Table 3.21 Kinetic modeling for Curcumin from NPs loaded Gel

Higuchi	Zero order	First order	K-peppas	Hixon crowel
0.10	-2.33	-0.57	0.956, n=0.188	0.267

The best fit drug release model in the case of gel formulation for both drugs was also Korsmeyer Peppas with values of n less than 0.5. this indicated Fickian diffusion in the case of gel formulations as well.

3.9 *In-vivo* Experiments:

In-vivo experiments in mice were planned and conducted as described in the chapter of methodology (2.13). The findings of all the behavioral experiments were obtained in triplicate and mean and standard deviation were plotted on graphs in excel. Various trends

were observed in different groups of animals and interpretation with respect to effectiveness of nanoparticle gel was measured by applying statistical formulae.

3.9.1 Findings of Behavioral experiments:

3.9.1.1 Mechanical allodynia:

The test for pain threshold measurement was conducted as described previously (2.14.1). Figure 3.20 describes the difference in pain threshold between the disease group and treated groups at the interval of 2 hours after application of gels and 6 hours after application of gels (curcumin-celecoxib gel, curcumin celecoxib NPs gel) One way ANOVA was applied to the results and p-value < 0.05 was considered statistically significant (p-value <0.05*,<0.01**,<0.001***).

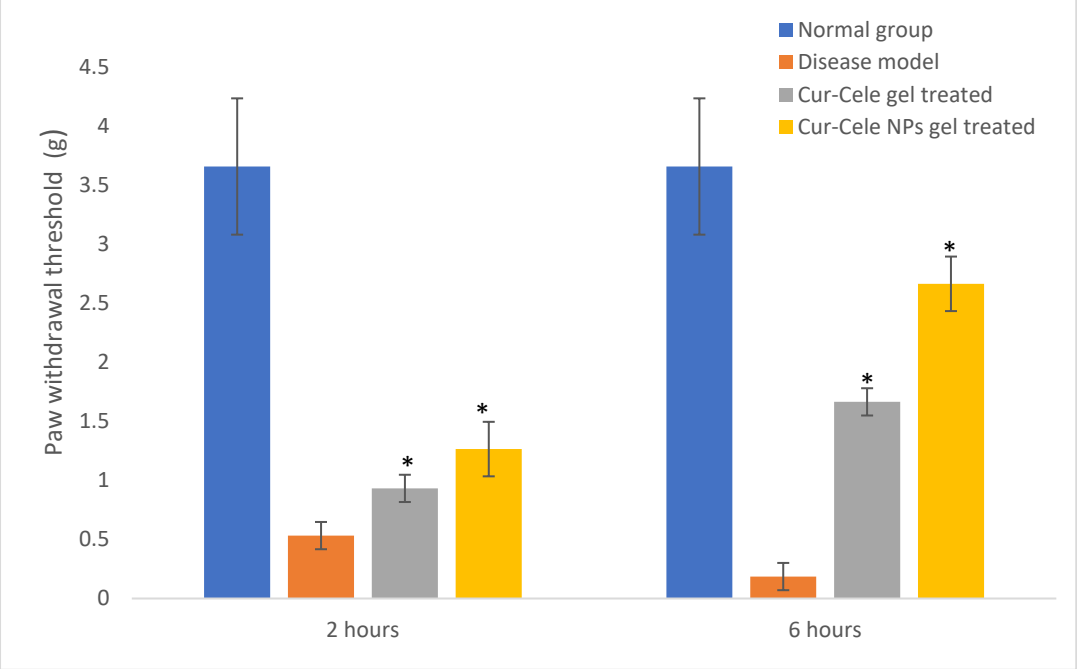


Figure 3.20 measurement of pain threshold with Vonfrey filaments *(n=3)*

3.9.1.2 Thermal Hyperalgesia

Hot plate test was conducted through the method previously described (2.14.2). Paw withdrawal latency period was calculated in seconds and the data were plotted on graph (figure 3.21).

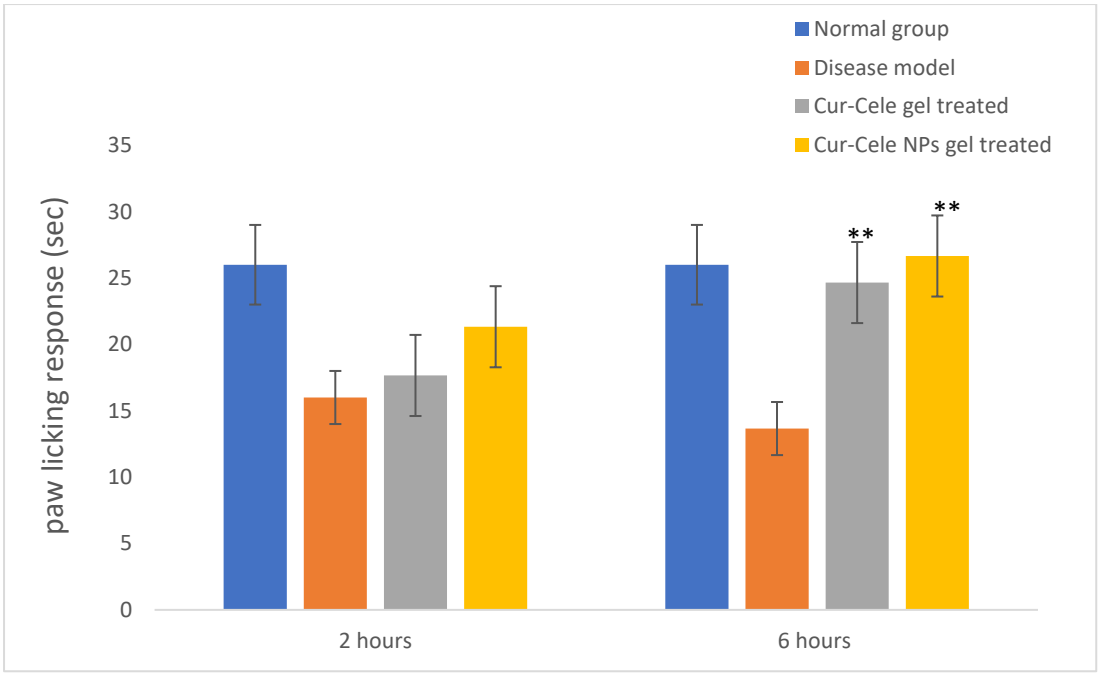


Figure 3.21; Results for thermal hyperalgesia test (hot plate method) (*n=3)*

3.9.1.3 Paw Edema measurement:

Paw edema was measured for three groups through method previously described (2.14.3). The groups included in this test were the negative control and two positive control groups.

Edema was calculated in millimeters and the data were plotted as graph in order to study the difference between diseased state (negative control) and treated groups (figure 3.22).

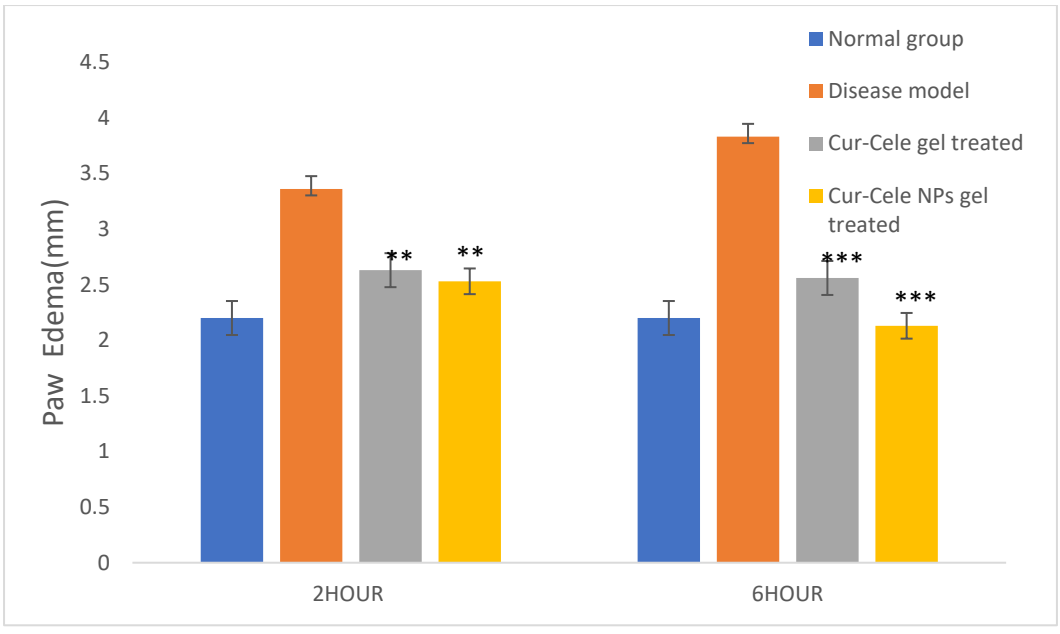


Figure 3.22; Results for paw edema measurement (*n=3)*

CHAPTER 4

DISCUSSION

4. DISCUSSION

OA is a chronic and progressive, inflammatory disorder of the joints. The worldwide prevalence of this disease is about 10% of men and 18% of women above the age of 65 affected by OA (Glyn-Jones *et al.,* 2015). The incidence of this disease is directly proportional to age (Ashford and Williard, 2014). The traditional treatment of OA is targeted towards the symptomatic relief in patients from pain caused by the ongoing process of inflammation (table.1.1). However, the chronic nature of this disease and the presence of co-existing medical conditions in patients of OA is responsible for limitations associated with current treatment regimen. These limitations include decreased patient compliance and more ADRs (table.1.2).Therefore, there is a need for dosage forms, designed to provide maximum anti-inflammatory activity while maintaining patient compliance, and reducing the incidence of adverse drug reactions (1.6).

To address this need, the present study focused on fabrication of curcumin and celecoxib co-loaded PLGA nanoparticles, for prolonged anti-inflammatory effect on arthritic joints. These nanoparticles after fabrication were loaded into CP-934 gels for easy topical application on the joints. Curcumin and celecoxib both are hydrophobic compounds with the limitation of decreased solubility associated with them. They possess strong anti-inflammatory properties and the synergistic combination of these drugs as an anti-inflammatory has been explored widely in literature (1.4.3). The method which was selected to fabricate PLGA nanoparticles was oil in water emulsification solvent evaporation method. This method is also considered the optimum method for fabricating nanoparticles of hydrophobic compounds. The process parameters which were studied and adjusted were defined by literature review and included amount of drugs, concentration of surfactant, stirring speed and ratio of ethyl acetate and surfactant (2.4.2.1). The effects of these parameters were studied on size of nanoparticles, entrapment efficiency of nanoparticles and polydispersity index.Literature survey confirmed that topical nano formulations with the size range between 20nm to 200 nm have better chances of penetrating stratum corneum and accumulating at target site of inflammation (Alvarez-Román *et al.,* 2004). The optimized nanoparticles with higher encapsulation efficiency possessed the size of approximately 220nm, hence smaller sized particles were successfully fabricated. These results were found to be consistent with PH sensitive nanoparticles

reported previously of curcumin and celecoxib drug combination in the case of inflammatory bowel disease (Gugulothu *et al*., 2014).

The use of drug combinations in nanoparticulate systems can be a cause of interactions at drug-drug and drug polymer level. It was therefore mandatory to explore the presence of interaction (if any) within the nanoparticle system of both drugs. FTIR studies were conducted of the polymer PLGA, drugs curcumin and celecoxib separately and then of the prepared formulation. Various functional groups were detected in this analysis which were expressed through different peaks at different wavelengths. This analysis showed that no functional group was shifted or removed and no presence of new bonds was detected. This test confirmed that there was no interaction at drug-drug or drug-polymer level, indicating the stability of the formulation. The results for this test were consistent with results of a previously reported stability indicating method for simultaneous determination of curcumin and celecoxib on High performance Liquid chromatography (HPLC) (Gugulothu and Patravale, 2012).

A higher encapsulation efficiency is a desirable requirement for nanoparticles. The process parameters that were found to effect encapsulation efficiency were stirring speed and amount of drug. It was observed that as stirring speed was increased the particle size and encapsulation efficiency decreased. The increase in amounts of both drugs led to increase in size and consequently increase in encapsulation efficiency (figure 3.8).

Zeta potential is a measure of stability of nanoparticulate dispersion. Generally, a vaue of +/-40 mv is an indication of good colloidal stability. The values of zeta potential obtained in this study for all formulations was upto -18 (3.2.6). Although this value does indicate some aggregation in nano formulation, however since the nano formulations were freeze dried before further use, the values of zeta potential did not pose any problem whatsoever.The effect of polyvinyl alcohol which was used as surfactant was also studied on particle size and it was found that an increase in the concentration of PVA solution led to a decrease in size of nanoparticles (figure 3.7). SEM analysis was carried out for the nanoparticle suspension and it was found that the particles possessed a very distinct spherical shape. Smaller particle size which was found earlier through zeta sizer, was also confirmed with SEM results (figure 3.9).

After successful fabrication of nanoparticles, they were loaded into CP-934 gels. Different concentrations of CP solution were prepared and gel matrix was achieved by addition of TEA. All of the different CP concentrations were studied for various parameters. These included, clarity, homogeneity, spreadability and PH as reported by literature (table 2.5).

A trend which was observed in regards to the increase in CP concentration was that, as it increased Spreadability was found to decrease (figure 3.13). This could be attributed to increase in viscosity due to increase in concentration of CP. The gel formulation with the best results in terms of clarity, homogeneity and PH was achieved with 1.5% of CP solution. The Spreadability value of this particular formulation was found to be 313mm^2. The viscosity of the optimized gel was calculated with Brookfield viscometer and was found to be 9277 cps. CP is known to formulate highly viscous semi-solids and our findings in case of viscosity value were consistent with those reported in case of CP gel reported in a previous study (Ubaid *et al*., 2016).

In order to study the flow behavior of the gels viscometer was used with varying speeds. It was observed that the increase in the speed of viscometer lead to more stress on the gel formulation which decreased the viscosity of the gel for that particular time. This shear thinning effect is a desirable property for semi solids as it increases the ease of application of gel formulations (Zeb *et al.*, 2017). The PH of all gel formulations was found to be between 4.5 to 6.5. This is considered a suitable PH range for topical formulations since the PH of skin is 5.5.

Stability is another requirement for gel formulations as it increases the shelf life as well as effectiveness of the semi solid. In order to study the stability three gel formulations loaded with drugs were kept in stability chamber for accelerated stability studies at 40C and 75% humidity. Stability studies were also carried out at room temperature. The results obtained from stability studies over a span of four months indicated that the gels exhibited good stability at room temperature however presence of clog and loss of viscosity was observed in accelerated stability testing after two months. A study was conducted to research the effect of shear, temperature and neutralizer on CP gel structure and it was concluded that increase in temperature has a destructive role in firm and strong structure of gel matrix. A decrease in temperature may cause the gel structure to regain its structure as CP has thixotropic property (Kim *et al.*, 2003). These findings were found to be consistent with the stability results obtained for nanoparticles loaded CP gel formulated in our study.

Drug release from nanosuspension as well as from nanoparticles loaded gel formulations was studied for celecoxib and curcumin. The results indicated burst release of both the drugs at the PH 5.5 which is the PH of the skin. Furthermore, it was observed that the drug release from gel formulation followed the same pattern as the drug release from nanoparticles. This indicated that no retardation of drug release was observed when drugs were loaded into gels. After the initial burst release the release of both drugs was slow and sustained over a period of 48 hours from both nanoparticles and nanoparticles loaded gels. (figure 3.18, figure 3.19).

The drug release kinetic models are utilized to explain the release of drugs and in this study a software DD- solver was utilized to apply kinetic release models. The highest values obtained were for Korsmeyer Peppas model for both drugs. The value of “n” was less than 0.5 in both cases which indicated Fickian diffusion. Fick's diffusion is the classical diffusion that is controlled by thermodynamic forces (by gradient of chemical potential or differences of concentrations) with the constant (or even pseudo-constant) diffusion coefficients and relatively simple kinetic profiles of penetrant redistribution in polymer matrix as the consequence of 1st and 2nd Fick's laws.

Permeability test for nanoparticles loaded gels were also conducted using Franz diffusion cell apparatus and permeability of nanoparticles loaded with drugs across the skin was calculated (table 3.15). A slow but continuous permeation of nanoparticles was observed, moreover the *in-vivo* evaluation confirmed that nanoparticles loaded gels have the potential to permeate in the skin and decrease inflammation.Permeation test was conducted because even though the formulation designed is not named transdermal, it’s local effect will be produced beneath the surface of the skin as the inflammation is underneath the skin in the case of OA (Figure 1.2).

The preliminary *in-vivo* experiments were carried out for the nanoparticles loaded gels in order to establish the effectiveness of the combination of drugs in nanoparticles loaded gels (2.13). Albino mice were utilized and acute model of inflammation carrageenan was used. The findings of the in-vivo experiments were found to be consistent with the release pattern of NP loaded gels. The two positive control groups were treated post carrageenan. One group was treated with plain hydro alcoholic drugs gel while other was treated with NP loaded gel. Behavioral tests were conducted (2.14) and the findings were

recorded in triplicate. Statistical analysis revealed highly significant differences among the disease induced untreated group and the two treatment groups of mice, in the case of mechanical allodynia and Paw edema measurement, while less significant differences in the case of hot plate test (figure 3.22, figure 3.23, figure 3.24). While exploring the reason as to why this occurred, it was proposed that due to the tests being performed in the cold climatic conditions (irrespective of the maintained laboratory temperature) the hot plate had lesser effect whatsoever on all the groups of animals.

Although the *in-vivo* tests conducted were of very preliminary nature due to the limitation of time, even so the results indicated the effectiveness of the formulation on diseased state via topical route. However a stronger medical evidence may be produced by conducting further *in-vivo* evaluation on the chronic model of inflammation such as CFA induced arthritis. Moreover while highlighting the significance of this piece of research it should be noted that this study is the first ever report on fabrication of curcumin and celecoxib PLGA NPs. It is also first piece of preliminary evidence of effectiveness of this drug combination against inflammation in the form of a topical gel.

CONCLUSIONS

- PLGA Nano particles of Curcumin and Celecoxib were successfully fabricated with smaller particle size and high encapsulation efficiency
- These Nano particles were then loaded into a novel formulation of Carbopol gels and various tests for characterization and release of Nano particles were conducted
- The release profiles indicate that the solubility of both drugs has markedly enhanced.
- Hence, we can safely conclude that PLGA Nano particles of Curcumin and Celecoxib loaded into topical gels could serve as an efficient carrier system to deliver the drug locally into the arthritic joint, while maintaining patient compliance and enhancing anti-inflammatory activity

FUTURE PROSPECTIVES

Further *in-vivo* testing could be conducted to further evaluate the synergistic action of Curcumin and Celecoxib nanoparticles loaded gels.

Extrapolation of *in-vivo* data on animal models could provide more information regarding the efficacy and safety of this dosage form designed.

As both of the drugs utilized in this study are FDA approved, *in-vivo* human trials could be designed in order to explore the potency and effects on human volunteers.

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