

**DEVELOPMENT AND EVALUATION OF TOPICAL  
NANOFORMULATION OF CHRYSIN AND  
PROBIOTIC FOR PSORIASIS**

A

Thesis

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In

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**LOVELY PROFESSIONAL UNIVERSITY**

**PUNJAB**

**2023**

## **DECLARATION**

I, hereby declared that the presented work in the thesis entitled “**DEVELOPMENT AND EVALUATION OF TOPICAL NANOFORMULATION OF CHRYSIN AND PROBIOTIC FOR PSORIASIS**” in fulfilment of degree of **Doctor of Philosophy (Ph.D.)** is outcome of research work carried out by me under the supervision of Dr. Sheetu working as Associate Professor in the department of Pharmaceutical Sciences, School of Pharmaceutical Sciences Lovely Professional University, Punjab, India. In keeping with general practice of reporting scientific observations, due acknowledgements have been made whenever work described here has been based on findings of other investigator. This work has not been submitted in part or full to any other University or Institute for the award of any degree.

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## CERTIFICATE

This is to certify that the work reported in the Ph.D. thesis entitled “**DEVELOPMENT AND EVALUATION OF TOPICAL NANOFORMULATION OF CHRYSIN AND PROBIOTIC FOR PSORIASIS**” submitted in fulfillment of the requirement for the reward of degree of **Doctor of Philosophy (Ph.D.)** in the department of Pharmaceutical Sciences, School of Pharmaceutical Sciences Lovely Professional University is a research work carried out by Shaik Rahana Parveen (11916564) is bonafide record of his/her original work carried out under my supervision and that no part of thesis has been submitted for any other degree, diploma or equivalent course.



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## Abstract

The present research study deciphers development and evaluation of topical novel gel containing Chrysin (CS) loaded nanostructured lipid carriers-probiotic (PB), i.e., *Bifidobacterium infantis* (BI) for psoriasis. CS is chemically 5,7 dihydroxy flavone, present in passion flower, bitter melon, wild Himalayan pear, Radix scutellariae, mushroom, Diaphragm *juglandis fructus*, walnut pellicle, flowers of *Juglans regia*, leaves and fruits of doumpalms (*Hyphaenethebaica*), Propolis, *Chaetomium globosum* fungus, green marine algae and *Cytisus villosus* Pourr, honey and Banxiaxiexin,

It has anti-angiogenic, anti-diabetic, anti-inflammatory, anticancer, neuroprotective, antioxidant, cardio protective and hepatoprotective activities. The mechanism of action of CS was reduction of tumor necrosis factor –alfa (TNF- $\alpha$ ), interleukin 17 A (IL-17A), interleukin-22 (IL-22) and antimicrobial peptides release (AMP) from epidermal keratinocytes. It downregulates the expression of PCNA (Proliferating cell nuclear antigen), COX2 (Cyclo-oxygenase-2), and maintains the cellular homeostasis. Moreover, activation of NF-kB pathway (Nuclear factor-kappa B pathway), phosphorylation of MAPK (Mitogen activated protein kinases) and JAK-STAT (Janaus kinase signal transducer and activator of transcription proteins signalling pathways) are also get attenuated hence, it exerted antipsoriasis activity. However, CS undergoes first pass metabolism when administered orally, and has poor bioavailability to prevent that effect, it was selected for topical route of administration after loading it in nanostructured lipid carriers' system (NLCs)

*Bifidobacterium infantis* UBBI-01 (BI) is the gram-positive probiotic. It was non-pathogenic microorganism available in market as of capsules, tablets and powder. It's clinical study in psoriasis patients reported that reduction of interleukin-6 (IL-6), C-reactive protein (C-rp), and "TNF- $\alpha$ " levels upon oral administration. In another clinical study it was confirmed that it has immunoregulatory effect by the induction of IL10 and also scurfin in the body. As per the clinical potential reports of the BI upon oral administration, it was hypothesised that a combinational approach might be aids in the management of the psoriasis

successfully upon topical administration. Hence to explore the CS and BI for psoriasis treatment in combinational manner NLCs based gel was developed. For that initially optimized CS loaded NLCs were developed to that PB was added, furthermore it was converted into the gel, by using the carbopol 940 as gelling agent

Modified hot homogenization method, followed by ultra bath and probe sonication (MHHM) was used to develop CS-loaded NLCs. To prepare the CS loaded NLCs, glyceryl monostearate (GMS) as a solid lipid (SL), capryol 90 (C-90) as a liquid lipid (LL), tween 20 (T20) as a surfactant (SR) and transcitol HP (THP) as a co-surfactant were selected based on solubility study. The components were optimized by applying 3 level 4 factor design Box Behnken Design (BBD). The average particle size (PS) and zeta potential (ZP) of optimized CS loaded NLCs were found to be  $60 \pm 2.312$  nm and  $-22.2$  mV respectively. To the optimized CS loaded NLCs, PB was added and then co-loaded in to the gel. The scanning electron microscopy (SEM) results of the CS NLCs-PB loaded gel represents spherical morphology and measured average size of the  $66 \pm 2.312$  nm, that complies with the results particle size analysis. In SEM report probiotic was appeared in “Y” and rod shape morphology.

The novel gel has shown the antibacterial activity against the *Staphylococcus aureus*. The zone of inhibition (ZOI) of CS-NLCs-PB loaded gel was 0.5, 0.2 and 0.54-folds higher than naive PB gel alone, naïve CS gel alone and CS-PB gel combination, respectively. The *in vitro* diffusion studies revealed that  $98 \pm 0.06$  % of CS got released from CS-NLCs-PB loaded gel at the end of 48 h. For initial 8h, release of CS was about 2.5 folds reduced in case of CS-NLCs- PB loaded gel than that of naive CS gel and thereafter the slow release was proceeded further, which indicated the sustained release of CS from NLCs.

The *ex vivo* permeability study of the CS NLCs-PB loaded gel has shown the  $28 \mu\text{g}/\text{cm}^2/\text{h}$  permeability, within 24 h, it was indicated that the formulation has shown the sustained effect when compared with CS gel permeability i.e  $50 \mu\text{g}/\text{cm}^2/\text{h}$  in 8h. The flux of the CS NLCs-PB loaded gel was  $1.024 \mu\text{g}/\text{cm}^2/\text{h}$ . The skin deposition

study of CS NLCs-PB loaded gel has shown the 2.8 folds higher ( $p < 0.05$ ) than the CS gel.

It was indicated that the developed formulation has the higher deposition of the CS on stratum corneum. The anti-psoriatic effect of the CS NLCs-PB loaded gel on imiquimod (ImQ) triggered psoriasis in mouse was produced by the reduction of psoriatic area severity index (Erythema, scaling, thickness of skin and ear). The histopathological studies of treatment groups revealed that the reduction of acanthosis, hyperkeratosis and inflammatory mediators. Furthermore, the reduction of TNF- $\alpha$  and induction of the IL-10 levels was confirmed by the ELISA test. The antioxidant effect of CS-NLCs-PB loaded gel was confirmed by the study of increment levels of catalase, SOD (superoxide dismutase), GSH (glutathione) and reduced levels of the MDA (Malondialdehyde). In addition to that the splenomegaly was also decreased upon topical application of the developed gel. It was concluded the novel composition containing CS NLCs-PB loaded gel successfully reduced the symptoms associated with the psoriasis and even it shown the dual pharmacological effect by the reduction of TNF- $\alpha$  and induction of IL-10 levels. The developed formulation didn't show any phase separation after the long term accelerated stability studies.

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## List of abbreviations

AMP	Antimicrobial peptides
AP-1	Activator protein 1 transcription factor
BI	<i>Bifidobacterium infantis</i>
BBD	Box Behnken design
CS	Chrysin
C/EBP	Protein family forms the transcription factor in controlling immune system responses
CCL20	Chemokine (C-C) motif ligand 20
cm <sup>-1</sup>	wave number
C90	Capryol 90
DC	Dendritic cells
°C	Degree centigrade
DSC	Differential scanning calorimeter
ERK	Extracellular signal-regulated kinase
EP	European pharmacopoeia
FT-IR	Fourier transform infrared spectroscopy
GMS	Glycerol monostearate
GSH	Glutathione
GSK-3β	Glycogen synthase kinase 3 beta
HR-TEM	High resolution transmission electron microscopy
IL	Interleukin
IKK	I-kappa B kinase
IL	Interleukins-12/23/22/17A/17F/21/10
INF-α,β and γ	Interferon α, β and γ
IL-17A	Interleukin-17A
IL-22	Interleukin-22
JAK/STAT	Janus kinase-signal transducer and activator of transcription proteins signaling pathways
LC	Langerhans cell

LLs	Liquid lipids
Mdc	Myeloid dendritic cell
MDA	Malondialdehyde
MAPK	Mitogen activated protein kinase
mg	Milligram
NF-Kb	Nuclear factor kappa-light-chain-enhancer of activated B cells
NLCs	Nanostructured lipid carriers
nm	Nanometer
PB	Probiotic
PCNA	Proliferating cell nuclear antigen
p38MAPK	Mitogen activated protein kinase
%	Percentage
% CDR	Percentage cumulative drug release
% T	Percentage transmittance
Pdc	plasmacytoid dendritic cells
rpm	Rotation per minute
Sec	Seconds
SOD	superoxide dismutase
SRs	Surfactants
SLs	Solid lipids
T20	Tween 20
THP	Transcutol HP
TNF- $\alpha$	Tumour necrosis factor-alfa
TLR7/8/9	Toll like receptors 7, 8 and 9
Th1 and Th17	T helper 1 and 17 cells
T	T-cells
TAK1	TGF-beta-activated kinase 1
$\gamma\delta$ T	Gama delta T-cells
$\lambda_{\max}$	Absorbance maxima
$\mu$ g	Microgram

UV	Ultraviolet
w/v	Weight by volume
XRD	X-ray diffraction method

## List of Appendices

S.NO.	Patent/Publication/book-chapter/webinar/ event
1	Copyright
2	Patent
3	Event
4	3 <sup>rd</sup> International Symposium Women and Girls in Science (2023)
5	3 <sup>rd</sup> International Conference (ICP-2022)
6	National Symposium on “Translational research and future Pharmaceuticals” (2022)
7	Two days International Conference on Advances in Pharmaceutical and Health sciences (APHS-2021) International Conference on Advances in Pharmaceutical and Health sciences (2021)
8	Research article
9	Research article
10	Book chapter
11	Research article
12	Research article
13	Review article
14	E-Symposium
15	Webinar
16	Webinar series no:2
17	Webinar
18	Short term course
19	Webinar

# Chapter 1



## Introduction

## 1.Introduction

Psoriasis is a genetic, non-infectious, autoimmune and chronic skin disorder (1). It occurs at any age (1) and its clinical manifestations include dry, itchy, erythematous silvery scales on scalp, limbs, knees, umbilicus, skin, face, feet, nails and intertriginous areas (2,3) The origin of psoriasis has not yet been, defined however at present it is manifest that it is related to heredity, deregulated immune system function, gut and skin dysbiosis (4). Psoriasis is associated with many comorbidities which include cardiovascular diseases, diabetes, obesity, dyslipidemia, depression, anxiety, suicidal ideation and inflammatory bowel disease etc (5-7)

As per World Psoriasis Consortium Day, on 29<sup>th</sup> October 2016, globally 125 million people have been reported with psoriasis (International Federation of Psoriasis Association). In 2016, WHO reported psoriasis prevalence in various countries, that varies from 0.09% - 11.4 % (8). Parisi *et al.*, on behalf of the Global Psoriasis Atlas, revised the prevalence rate of psoriasis systematically in 2020. According to this report, there were 1.5 % cases in “North America, 1.92 % cases in “Western Europe”, 1.83 % cases in “Central Europe”, 0.14% cases in “East Asia”, 1.99 % cases in “Australasia”, 1.10 % cases in “Southern America” and 2.8 % cases in India. The higher prevalence rate has been reported in higher latitudes, mostly in white people as compared to other population (9,10). Psoriasis has negative impact on physical, social, mental well-being of patients which leads to socioeconomic burden on their life. In addition to that, too many people are living with psoriasis due to delayed diagnosis, lack of awareness and inadequate treatment options (11). Psoriasis can be divided into mild, moderate and severe based on the severity of disease. Different types of psoriasis categorised into plaque, pustular, inverse, guttate, and nail psoriasis (12).

Mostly 80-90% of people were affected with plaque psoriasis (13). The treatment strategies for psoriasis are selected based on the severity of disease and extent of affected body surface area. Topical therapy (emollients, keratolytics, coal tar, dithranol, corticosteroids and vitamin D3 analogues) is opted for mild psoriasis condition, whereas phototherapy (UV A and B), biological therapy



(Methotrexate, acitretin, cyclosporin, apremilast etc) are selected for moderate to severe. Despite several treatment strategies, psoriasis still remains incurable and persistent. Additionally, above mentioned treatment options are associated with many side effects which include skin thinning, skin irritation, burning, itching, red skin, inflammation of hair follicle melanoma, teratogenicity, hepato-toxicity, nephro-toxicity, hypertrophy and hair thinning etc (15,16). Therefore, it is important to find an alternative therapy that can overcome above mentioned side effects as well as ensure safety and efficacy. Ease of availability, affordable cost, fewer side effects and ability to act on multiple sites have shown increased demand in exploration of herbal drugs (17)

There are many herbal drugs that have been explored to treat psoriasis in the form of extract or isolated bioactives. Some of them include *Wrightia tinctoria extracts* (18) baicalin (19), *Baixuan Xia Ta Re Pian* (20), genistein (21), *Punica granatum L* (22) and Convallatoxin (23) isolated bioactives. Although herbal drugs are safe and effective but they show similar physicochemical problems as synthetic drugs which include insolubility, instability and poor bioavailability. In that scenario, physicochemical and physiological obstacles of herbal drugs can be overcome using a variety of novel drug delivery-based strategies (24).

The topical drug delivery approaches are mostly preferred due to their direct targeting effect and elimination of systemic side effects. The existing topical conventional approaches include gels, creams and ointments. These are associated with many limitations which include atrophy, high dosing frequency, drug leaching, irritation, burning, “less penetration of active agents” in the epidermis, poor retention capacity, and poor patient’s compliance (25). To overcome the above-mentioned challenges and improve the benefits, novel drug delivery-based approaches are suitable. These include liposomes, niosomes, micro emulsions, transfersomes, ethosomes, emulsomes, ethosomes, emulsomes, invasomes, dendrimers, nanoparticles and hydrogels etc (26) .

Additionally, a topical novel drug delivery system-based strategy improves the safety, efficacy of drug moiety and also patients’ satisfaction (27).

The explored herbal and synthetic topical novel approaches so far to treat psoriasis, include micro emulsions [methoxsalen (28), betamethasone dipropionate (29) tacrolimus (30) cyclosporin (31)], nano emulsions [cyclosporin (32) methotrexate (33)], nanoemulgel [betamethasone dipropionate (34), Leflunomide (35 ) ethosomes [psoralen (36) curcumin (37)], dendrimers (Dithranol (38)), liposphere gel (curcumin (39) thymoquinone and tacrolimus (40)], niosomal hydrogel [8-methoxy psoralen (41), niosomes (methotrexate (42), acitretin (43), liposomes (fusidic acid) (44)], polymeric nano spheres [Vitamin D3 (45)], solid lipid nanoparticles loaded in hydrogel [methotrexate and etanercept (46)] and nanostructured lipid carriers [(methotrexate and Triamcinolone (47)]

Although many synthetic and natural drugs have been loaded into NDDS, still some of the potential natural active moieties have not been formulated into dosage forms. Among them *Chrysin* (CS) is one such flavonoid. CS is ubiquitous in nature; abundantly present in mushroom (*Pleurotus ostreatus*), propolis, blue passion flower (*Passiflora caerulea*), honey and *Radix scutellariae* (48). CS has many potential pharmacological activities which include neuroprotective, antiviral, anti-asthmatic, antidepressant, antibacterial, anti-inflammatory, antiarthritic, anti-cancer, antiangiogenic, antimetastatic, immunomodulatory, and anti-apoptotic (49). It exerts antipsoriatic effect by regulating “I- kappa B kinase” (IKK) “MAPK”, “JAK-STAT”, and NF-kB “signalling pathways” (49,50). It leads to decreased levels of IL-22 induced CCL20, TNF- $\alpha$ , IL-17 A, and antimicrobial peptides release from psoriatic skin epidermis. Moreover, CS inhibits reactive oxygen species (ROS) production (50)

Despite the above-mentioned pharmacological actions of CS, it was associated with some of the limitations which include poor aqueous solubility and pre systemic metabolism (51). However, CS formulation development was not explored through topical route for psoriasis. Considering the aforementioned multiple mechanisms of CS towards management of psoriasis, it was anticipated to develop the topical formulation containing CS, which could avoid the first pass effect of it. As per literature survey a close interrelation was observed between psoriasis and dysbiosis of skin. The skin dysbiosis is the one of the sources for psoriasis, it could be due to the

presence of higher proportion of the pathogenic microbes and lower amount of commensal on the psoriatic regions. Numerous researchers have found an exacerbated count of *Staphylococcus* and *Streptococcus* bacterial pathogens on psoriatic skin (52,53). In addition, decreased levels of *Actinobacteria*, *Macrocooccus*, *Propionibacterium granulosam*, *Staphylococcus epidermidis* and *Propioni bacterium acnes* have also been reported on psoriatic skin (54,55). As per the clinical study conducted by Salma *et al.*, in 2022 for psoriasis and non-psoriasis persons, reported that the higher colonization of the *Staphylococcus aureus* due to the impaired epithelial barrier function. The higher expression of IL-36  $\alpha$  and IL-17 A inflammatory mediators was observed, which caused skin inflammation, and it was further confirmed by real time quantitative PCR study (56)

In gut dysbiosis, damaged barrier increased the permeability, which causes the dissemination of intestinal microbiota and their metabolite (SCFA, secondary bile acids, tryptophan, lipo-polysaccharides, p-cresol and phenol) from gut into skin through systemic circulation” (55). It further leads to epithelial barrier disruption, impairment of skin homeostasis and altered innate and adaptive immune system, overall, these factors promote the progression of psoriasis (55).

Considering the aforementioned skin and gut dysbiosis, PB are thought to be suitable therapeutic agents in the management of psoriasis. In one of the studies probiotics have been reported for amelioration of the skin inflammation in psoriasis. For instance, clinical study of *Bifidobacterium infantis* 35624, upon oral administration reduced the “TNF- $\alpha$ ”, “IL-6” and serum “c- reactive protein (c-RP)” levels in the body (57). In 2017, Chen *et al.*, reported the reduced levels of “IL-23”, “IL-17A/F”, “IL-6”, “IL-22” and “TNF- $\alpha$ ” in ImQ triggered psoriasis mice when “*Lactobacillus pentosus* GMNL-77” (58) probiotic administered orally. The probiotics were found to reduce (58). Rather *et al.*, in 2018 extracted the SEL001 from *Lactobacillus sakei* *probio 65* probiotic and evaluated antipsoriatic activity in IMQ triggered psoriasis like inflammation in mouse model. This study reported about reduction of IL-19, IL-17 A and IL-23 upon topical administration of SEL001 (59)

Looking at the potential activity of probiotics, it was intended to develop a topical nano delivery system containing CS and probiotics. Among various topical NDDS, it was proposed to explore NLCs to deliver CS and probiotics in the form of gel.

NLCs are one of the superior drug carrier systems over the other topical forms due to their high drug loading capacity and prolonged release effect at targeted site. NLCs could be potential, safe and biocompatible drug delivery system along with enhanced skin permeation and retention (60). In addition to that, topical NLCs formulation could avoid challenges associated with psoriasis. These include imbalanced lipids, decreased levels of ceramide, dry skin and poor penetration of drug via stratum corneum. Hence loading of CS into nanocarriers would overcome these challenges.

Some of the studies where NLCs have been successfully explored in psoriasis by topical administration include methotrexate, dithranol and mometasone furoate. Agrawal *et al.*, in 2020 formulated and evaluated, methotrexate loaded NLCs gel. NLCs were able to improve the bioavailability and reduce the systemic side effects of methotrexate (61). Sathe *et al.*, in 2019 formulated dithranol loaded NLCs gel and reported that NLCs hydrated the psoriatic skin due to the presence of SL and LL in NLCs formulation (62). Kaur *et al.*, developed NLCs based hydrogel of mometasone furoate in 2018. Their results have shown that the developed NLCs have desired stability and shown significant reduction of psoriatic lesions (63)

Based on the aforementioned advantages of NLCs over other topical delivery systems and multifunctional potential of CS, dysbiosis management and antibacterial potential of BI, the proposal has been made to develop a novel nano formulation gel for topical treatment of psoriasis. In order to achieve this objective, the CS NLCs-PB loaded gel was developed and evaluated for its antipsoriasis potential on ImQ induced mice.

# Chapter 2



## Review of literature

## **2. Psoriasis**

Psoriasis has an involuted multifactorial etiology (64), in which life style, drugs, skin dysbiosis, gut dysbiosis, genetic, stress, diet, and environmental factors interact with each other that leads to generation of psoriasis. It can occur at any part of the body and its condition differs from mild to moderate to severe types (64).

The NPF (National Psoriasis Foundation) classifies mild, moderate and severe psoriasis based on its severity and scaling scores (64).

### **2.1 Classification of psoriasis**

#### **2.1.1 Mild type psoriasis**

Less than 3% of the body surface area (BSA) gets affected

#### **2.1.2 Moderate type psoriasis**

Almost 3-5% of the BSA gets affected

#### **2.1.3 Severe type psoriasis**

It affects more than 10% of the body

### **2.2 Types of psoriasis**

There are different types of psoriasis such as inverse, guttate, pustular, plaque, and erythrodermic (65) which are discussed briefly below it

#### **2.2.1 Plaque Psoriasis**

It is a main type of psoriasis and covers 80-85% on elbows, knees, lower back and scalp in the form of red coloured spherical lesion which further develops into patches and silvery plaques (65,66).

##### **2.2.1.1 Treatment**

Topical, phototherapy and systemic medications are given alone or in combination.

#### **2.2.2 Guttate Psoriasis**

It appears as a minuscule dewdrop size (10 percent) around arms, legs, trunks and scalp. It occurs at the age of childhood and progresses highly in adult's age.

### **2.2.2.1 Treatment**

UVB phototherapy or oral treatments is the first line treatment. If UVB phototherapy is not available, guttate psoriasis persists. Then a combination of biologics treatments is preferred.

### **2.2.3 Inverse Psoriasis**

It occurs in the skin folding with red and shiny appearance.

**2.2.3.1 Treatment:** Topical treatment is recommended (1% hydrocortisone, calcipotriene, coal tar or anthralin)

### **2.2.4 Pustular Psoriasis**

It mainly effects to soles and palms, filled with pus, sometimes nail also gets affected.

#### **2.2.4.1 Treatment**

Acitretin, cyclosporine, methotrexate and TNF- $\alpha$  blockers are used.

### **2.2.5 Erythrodermic Psoriasis**

It affects the whole body and it shows the symptoms like redness, itching, pain and inability to regulate body temperature.

#### **2.2.5.1 Treatment**

Systemic drugs are recommended

## **2.3 Pathogenesis of psoriasis**

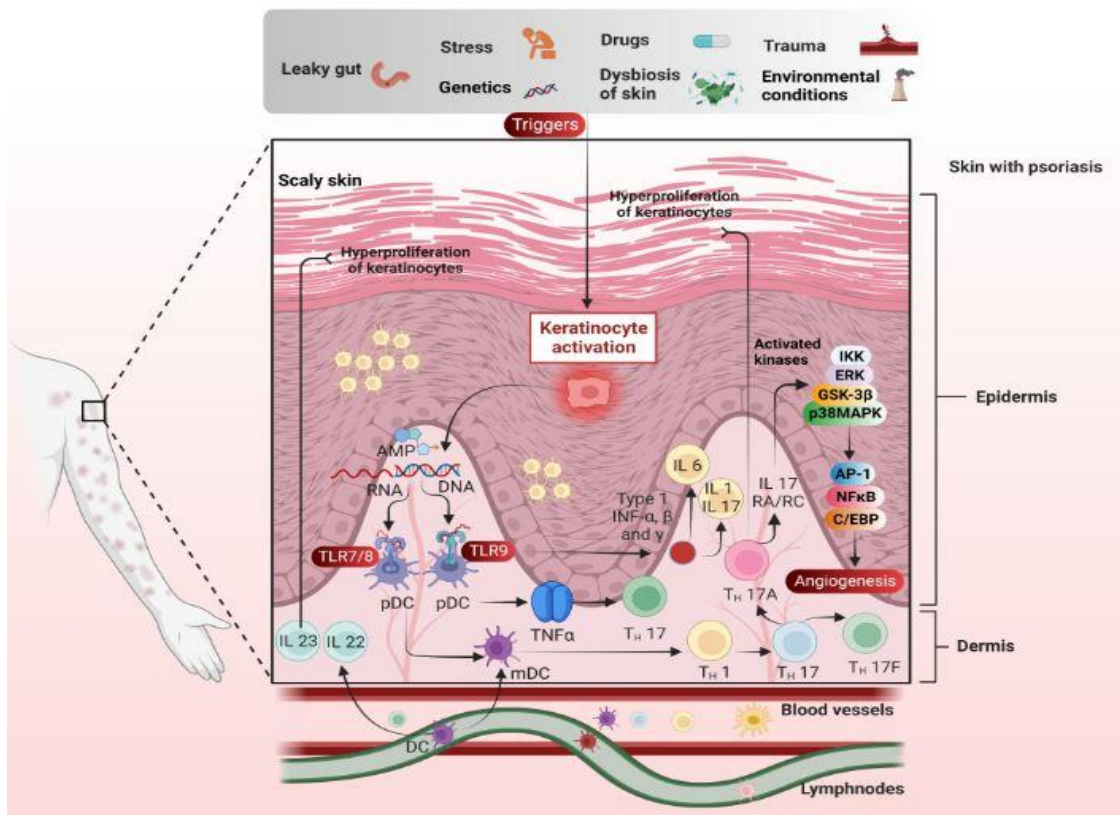
Pathogenesis of psoriasis is unclear; however, the existing literature review shows that it can occurs due to increased proliferation of keratinocytes. It is a multifactorial skin disease which is triggered by genetics, environmental changes, skin injury, drugs, trauma, stress, leaky gut and skin infections (increased *Staphylococcus*, *Corynebacterium*, *Propionibacterium* and *Streptococcus*). This leads to synthesis of antimicrobial peptides (LL37,  $\beta$ -defensins and S100) from damaged keratinocytes of skin (66). LL37 complexes with self-DNA are produced from damaged cells. The complex stimulates the toll-like receptors (TLR-9) in plasmacytoid dendritic cells (pDC) (67). The activated PDC produces type -1 IFN (INF- $\alpha$ & $\beta$ ). Type-1 IFN signalling promotes myeloid dendritic (mDC) cells phenotype maturation, shows

differentiation and function of IFN- $\gamma$ , Th1 and Th17. It leads to the generation of IL-17, TNF- $\alpha$ , IL-1 and IL6. LL37 also binds RNA and stimulates, pDC of TLR7. The complex of LL37-RNA acts on mdc (myeloid dendritic cell) of TLR-8 and it activates mdc. Activated mdc transfer into lymph nodes and develops more amount of “TNF- $\alpha$ ”, “IL-23”, and “IL-12” (67). This modulates differentiation and proliferation of Th17 (T-helper 17) and Th1 (T-helper1). The Th17 cytokines, namely IL-17, 21 and 22 activate Keratinocyte proliferation in epidermis. Further, the TNF- $\alpha$ , IL-23 and Th-17 cause inflammation that led to plaque psoriasis.

The “IL-17” is consists of 6 members of “IL-17A-F”. IL17A binds to trimeric receptor and form adaptor protein ACT1. The binding of ACT1 and the IL-17 activated the “glycogen synthase kinase 3 beta” “(GSK-3 beta)” “p38 MAPK”, “extracellular signal-regulated kinase” (ERK), “TGF-beta-activated Kinase 1” (TAK1), and “I-kappa B kinase” (IKK). These “kinases stimulate “NF- $\kappa$ B”, “chemokines”, AMP, “AP-1” and “C/EBP” transcription of pro-inflammatory cytokines”, (66,67). “Th1 and Th2 cytokines acts via JAK-STAT signalling pathways”. The drugs which target “IL-17” “TNF- $\alpha$ ”, “IL-23”, and “JAK/STAT” pathway are potential for plaque psoriasis therapy” (66,67).

The cascading events of immune pathogenesis are illustrated in **Figure 1**. The key role in psoriasis is triggered by the NF- $\kappa$ B, which provide maximum transcription of those cytokine, so primary objective of the treatment of psoriasis is inhibition of TNF- $\alpha$ , NF- $\kappa$ B, IL-17A which can ultimately prevent the remission of the disease. Another cause of psoriasis is absence or dys-regulation of T-regulated cells (e.g. IL-10).





**Figure 1.** Sequences involved in the immune pathogenesis of psoriasis

## 2.4 Current treatment options for psoriasis

Psoriasis is multifactorial and no exact treatment is proposed for it. The treatment starts from conventional topical agents to biologics. Unfortunately, the existing conventional treatment options i.e., topical therapy and phototherapy are able to control the psoriasis but do not treat it completely. The biological therapy is more effective in psoriasis treatment; however, it causes many side effects and treatment is more costly.

**2.4.1 Topical therapy** the selection criteria of treatment route are based on the severity of the psoriasis condition. The mostly employed route for mild to moderate condition is topical and for "moderate" to "severe" is oral or injectables. The topical treatment options include anti-inflammatory agents, immunomodulators, antihyperproliferating agents and immunosuppressants. **Table 1** describes the conventional topical treatment options, their mechanism of action and side effects

**Table 1. Conventional topical treatment options for psoriasis**

<b>Drugs</b>	<b>Mechanism of action</b>	<b>Side effects</b>	<b>References</b>
Psoralen plus ultraviolet A exposure (PUVA)	Inhibits replication of DNA Arrest cell cycle	Skin cancer	(68)
Ultraviolet B irradiation (UVB)	Apoptosis, immune-suppression	Burning and itching	(69)
Retinoids	Control proliferation and differentiation of keratinocyte	Skin redness	(70)
Glucocorticosteroids	Anti-inflammatory, anti-mitosis, apoptosis, vasoconstriction and immune-modulation	Degenerating and thinning of epidermis and dermis	(71)
Vitamin D3 derivatives	Regulates keratinocyte proliferation	Tingling	(72)
Keratolytics	Exfoliating hyper keratotic skin	Skin redness	(73)
Coal tar	Inhibits keratinocyte proliferation	Colouring of skin	(74)
Calcineurin inhibitors (Tacrolimus and pimecrolimus)	Inhibits the T cells & proinflammatory cytokines	Burning sensation and skin irritation	(75)
Dithranol	Anti hyper proliferative activity, Immune regulation and inhibits granulocyte function	Skin redness	(76)

### **2.4.2 Systemic therapy**

In systemic therapy, drugs were given orally and through intravenous route. The details of oral treatment options their mechanism of actions and side effects were shown in Table 2 (77).

#### **2.4.2.1 Biologics**

The recent advancement towards treatment of psoriasis is biologics (based on recombinant DNA technology) which shows anti-psoriatic activity by the reduction of TNF- $\alpha$ , IL-17 and 23 cytokines. It shows efficacious effect when compared with other classes of anti-psoriatic agents, however they are too expensive. The lists of biologics for treatment of psoriasis are given in Table 3 (84-85).

**Table 2. Oral treatment options for psoriasis**

<b>Drug Name</b>	<b>Mechanism of action</b>	<b>Side effects</b>	<b>References</b>
Apremilast	Cyclic AMP and phosphodiesterase - 4 inhibitor	Headache, tinnitus and naso-pharyngitis	78
Tofacitinib	Janus kinase inhibitor	upper respiratory tract infections, Naso-pharyngitis, diarrhoea, headache, hypertension, increased cholesterol levels, gastroenteritis, anemia, nausea, serious infections, and thromboembolism	79
Cyclosporine (Calcineurin inhibitor )	Calcineurin inhibition leading to reduced IL-12	Nephro toxicity and hypertension	80-81
Methotrexate	Anti metabolite Dihydro-folate reductase inhibitor	Hepatotoxicity and Nephrotoxicity	82
Acitretin (Retinoids)	Controls keratinocyte proliferation	Teratogenicity effect, hair loss, cheilitis and abnormal lipid level	83

**Table 3. Biologics for psoriasis**

<b>Biologics</b>	<b>Mechanism of action</b>	<b>Side effects</b>	<b>References</b>
Etanercept Adalimumab Infliximab Certolizumab Golimumab	Inhibits- TNF- $\alpha$	Injection site reactions, Naso pharyngitis and upper respiratory infections	(86)
Secukinumab Ixekizumab Brodalumab	Inhibits IL-17	Crohn's and ulcerative Colitis	(87)
Guselkumab	Inhibits IL-23	Back pain, diarrhoea and Tinea infections	(88)
Ustekinumab	Inhibits IL-12 and IL-23	Injection site reactions, cold, sore throat and diarrhoea	(89-90)

### **2.4.3 Phototherapy**

Phototherapy in the form of narrow band ultra violet B (UV-B), ultraviolet A1 (UVA1) and PUVA is used for treatment of psoriasis. The exact action in the reduction of psoriasis by phototherapy is not elucidated. However the existing data shown that UVB induce the apoptosis of T-lymphocytes and keratinocytes which leads to the reduction of hyperproliferation of psoriasis. UVA1 inhibits the activity of antigen presenting cells and langerhan cells their by it acts by showing immunosuppression effect. Although phototherapy has shown the better effect in the psoriasis treatment but its long term usage leads to cutaneous oncogenesis, vertigo, erythema, and cataract. (91)

The above-mentioned current treatment options were associated with many side effects. In that scenario it is important to find an alternative therapy. The literature survey has shown that the extracts and secondary metabolites from the plants have potential to treat psoriasis due to their safety and synergistic activity. Phytomedicines can reduce the side effects of synthetic agents such as atrophy, organ toxicity, immunosuppression, and carcinogenicity. The reported *in vitro* and *in vivo* studies for few herbal extracts and isolated pure constituents has shown its potential role in effective management of psoriasis. The detailed information about some herbal extracts and the isolated phyto constituents are given in Section 2.5 and 2.6 (92)

## **2.5 Herbal extracts for psoriasis**

### **2.5.1 BaiXuan Xia Ta Re Pian (BXXR) extract**

It is an ancient traditional uighur prescription medicine in China. Some of the phytoconstituents reported in the extract are *Euphorbiae humifusae herba*, *Chebula fructus*, *Terminalia belliricae fructus*, *Chebulae fructus immaturus aloe* and *Resina Scammoniae*. Xiaobo. Pang *et al.* in 2018 performed antipsoriatic effect on IMQ triggered psoriasis in mouse model. This study has shown the inhibitory effect of the BXXR extract on IL-17 signalling pathway and reduces inflammation (92,340)

### **2.5.2 *Tripterygium wilfordii* Hook.f. (TwHf) root decoction and extract**

It is a Chinese vine plant belonging to the family Celastraceae. It mainly consists of triptolide, celastrol, and demethoxyzeylasteral as active ingredients, which are

obtained from the root of TwHf. Ru *et al.* 2020 explored the antipsoriatic activity of TwHf decoction on IMQ triggered psoriasis model. It was found to reduce the skin inflammation, inhibit the keratinocyte (KC) multiplication and increased the apoptosis factors. Subsequently it acts on the T-lymphocyte release, which causes reduction of the cytokines (93,340).

### **2.5.3 *Punicagranatum* Linn**

*Punicagranatum* Linn of Lythraceae family was reported to have some active components such as *Punicalagin*, *2,3(S)-hexahydroxydiphenoyl-D-glucose*, *Punicalin*. Jacob *et al.* 2019 evaluated the *in vitro* anti-psoriatic activity of aqueous acetone extract of *Punicagranatum* fruit rind using thymidine phosphorylase inhibition assay. The isolated compounds showed the inhibitory activity against thymidine phosphorylase, but further *in vivo* evaluation required for confirmation of activity (94,340)

### **2.5.4 *Wrightia tinctoria* (Roxb)**

It is a traditional system of Indian medicine used for the management of skin diseases which include psoriasis (95). It belongs to the family of Apocynaceae. The extract and oils prepared from the leaves of this plant has shown the anti psoriatic effect due to the presence of flavonoid, glycoflavones-isoorientin and phenolic acids. Dhanabal *et al.* in 2012 evaluated the antipsoriatic activity of hydroalcoholic leaves extract of *Wrightia tinctoria* in mouse tail model (96) then reports antioxidant effect and reduction in orthokeratosis. Sundarrajan *et al.* in 2017 investigated the action of *Wrightia tinctoria* in the amelioration of psoriasis. They reported the 67 compounds and their 238 target proteins. Among these potential proteins, mostly targeted proteins are "apolipoprotein E" (APOE) (T21), "IL12B" (T123), "CAT" (T46), and "TP53" (T228) (97). Some of the compounds such as  $\alpha$ -sitosterol and cholesterol controls APOE protein for the regulation of lipid metabolism. Tryptanthrin inhibits mutant p53 for the regulation of apoptosis and cell division. Benzoic acid, 3-hydroxy-1-methylpropyl ester (C27) are reported to directly inhibit IL-12 (T123). Pyrogallol directly acts on CAT (T46) enzyme for the suppression of humoral immune mediated responses. It also activates the apoptosis by the induction of "Caspase 3" (T43) and "Caspase 8" (97) (T44). The synergistic mechanism of these compounds suppresses

the immune system and restores the disrupted apoptosis mechanism. This multi-targeted mechanism has shown its effect in treatment of psoriasis (97,340)

## **2.6 Isolated phyto constituents for psoriasis**

### **2.6.1 Luteolin**

*Luteolin* is a natural flavonoid obtained from the leaves of *Reseda luteola* belonging to Resedaceae family. *In vitro* studies on HaCaT cell line has reported reduction in IL-6, IL-8, vascular endothelial growth factor, and inhibited NF-kB activation. It also prevented proliferation of keratinocytes without effecting ATP production. Therefore *luteolin* has antipsoriasis effect (98,340)

### **2.6.2 Baicalin**

Baicalin has shown antiapoptotic, anti-inflammatory and antioxidant effects. Topical application of baicalin on IMQ induced psoriasis model has shown anti-inflammatory activity. It was reported to block the activities of cytokines, hence, reduction in acanthosis, neutrophil infiltration, hyperkeratosis, epidermal hyperplasia was observed showing its potential to treat psoriasis (99-100)

### **2.6.3 Convallatoxin (CNT)**

CNT is obtained from wild plant *Convallariamajalis* Linn (Asparagaceae) (101).

Bo-Wen Jiang *et al.* 2020 demonstrated potential activities of CNT via *in vitro* and *in vivo* studies. *In vitro* HaCaT cell line study confirmed necroptosis inhibition via ROS mediated mechanism. *In vivo* study on IMQ, 12-O-tetradecanoyl-phorbol-13-acetate (TPA) triggered psoriasis model demonstrated the inhibitory effect of CNT in the cytokines release which includes "IFN  $\gamma$ " from "Th1/Th17 cells", "IL-17F", "IL-17A", "IL-22", and "TNF- $\alpha$ " (101,340)

### **2.6.4 Rottlerin**

It is a poly-phenolic compound obtained from *Mallotus philipinens* is belonging to Euphorbiaceae family. Min M *et al.* 2017 has performed the "*in vitro*" and "*in vivo*" studies of *Rottlerin*. *In vitro* HaCaT cell line studies demonstrated the inhibition of keratinocyte proliferation by arresting NF-kB signalling mechanism. In additions to this it induces apoptosis via autophagy mediated pathway. In IMQ induced psoriasis

model it was reported to suppress chronic T cell dependent skin inflammation as well as inhibits cytokines. It also reduces skin thickening and angiogenesis (102,340)

### **2.6.5 PSORI-CM02**

It is Chinese herbal medicine formula, composed of *Rhizomacurcumae*, *Radix paeoniaerubra*, *sarcandra glabra*, *Rhizomasmilacisglabrae* and *Fructusmume*. Chen *et al.* 2018 conducted HaCaT cell line study which inhibited the cell proliferation at G1 phase. In IMQ induced psoriasis model study, it has reported to inhibit the TNF- $\alpha$ , IL-6 and IL-17 levels via NF-kB signalling pathway. This study also reported to control the oxidant/antioxidant status, alter the equilibrium between Th17 response and CD4+FOXP3+ Treg generation. Yue L *et al.*, 2019 investigated the effect of PSORI-CMO2. It induces the autophagy by suppressing the PI3K /Akt, mTOR and signal transduction pathway in the skin (103,340)

### **2.6.6 Genistein**

Genistein is a flavonoid extracted from the *Prunus* and *soy bean* belonging to the family of Legumes. Wang *et al.* 2019 conducted *in vivo* study on IMQ triggered psoriasis mouse model. The study has demonstrated to inhibits the "IL-1", "IL-6", "IL-17", "IL-23", "TNF- $\alpha$ " and "chemokines ligand 2" (CCL2) cytokines via "NF-kB pathway" (104,340)

## **2.7 Conventional herbal drug delivery system**

### **2.7.1 Fish oil soft-gelatin capsule**

Bittiner *et al.* in 1988 conducted 8-week clinical trial on 28 patients with the chronic stable psoriasis. The patients were grouped and subjected to treatment with fish oil capsules and placebo capsules daily along with a strict diet. A participant receiving fish oil therapy experienced a substantial reduction in psoriatic features, while the placebo group experienced no improvement in psoriasis symptoms (105,106)

### **2.7.2 Aloe vera (AV)**

AV gels, shampoos, organic extracts and creams were available in the management of psoriasis. It acts by water preservation in tissues of skin, remove dead skin cells, and promote the development of healthy skin (106-107).

### **2.7.2.1 AV cream**

Syed *et al.* in 1996 performed a clinical trial to assess the acceptability and effectiveness of topically applied AV extract cream for 16 weeks. It was found that the cream does not show any adverse reaction. The cream has shown significant improvement in the plaques of psoriasis (107,108)

### **2.7.2.2 AV gel**

Dhanabal *et al.* in 2012 tested AV gel for antipsoriatic effect. They found that the extract caused epidermal changes similar to tazarotene (0.1 percent) standard gel. AV contains lignin which primarily penetrates into deep skin layers that helps to cure psoriasis (109,340)

### **2.7.3 Curcumin gel**

Sun *et al.* in 2013 formulated gel containing curcumin and evaluated its anti-inflammatory activity on IMQ psoriasis model. *Curcumin* gel therapy greatly reduced the cytokine (110,340)

### **2.7.4 Silymarin gel**

Khan *et al.* in 2014 used Carbopol as a gelling agent to develop a topical gel of *Silymarin*. The gel has shown 96.30 percent of the drug release (111). The clinical skin irritation studies were conducted after adequate *in vitro* parameters, and their results show no sign of irritation at the end of 72 hours (112,340)

### **2.7.5 Nigella sativa capsules and ointments**

Jawad *et al.* in 2014 performed a randomised clinical trial in 60 psoriatic patients to assess the effectiveness and safety of *Nigella sativa* (NS) when given orally (capsule) and topically (ointment). The outcomes of the study were evaluated using a PASI score and MDA levels (113,340).



When the PASI was calculated, it was revealed that 65 percent of the ointment group has fully healed from psoriatic lesions, while 50 percent of the oral administration group was healed. However, better results were obtained with the combination treatment where 85 percent lesions were healed. The Serum MDA levels were measured by thiobarbituric acid assay before and after 12 weeks of treatment. In patients using the ointment of NS (10 percent w/w) twice daily,  $0.78\pm 0.23$   $\mu\text{mol/L}$  baseline serum MDA level was observed. The level of MDA in patients received with powder NS 500mg capsule was  $1.09\pm 0.37$   $\mu\text{mol/L}$ . The level of MDA in patients with combination treatment was  $1.2\pm 0.37$   $\mu\text{mol/L}$ . The MDA levels of psoriatic patients decreased in all three categories as comparison to pretreatment levels ( $p < 0.05$ ) (113,340).

Looking at the beneficial effects of the phytomedicines in psoriasis treatment and their ability to act at a time on multiple mechanisms of the body recommended a new insight in exploration of herbal, animal and microbial pure constituents into dosage forms. This path of development started receiving more advantages. Along with above mentioned phytomedicines, some of the pure compounds derived from the plants were subjected to animal studies are summarized below in Table 4 (114).

**Table 4. Preclinical evaluation of herbal drugs in psoriasis**

<b>Herbal drug</b>	<b>Evaluation</b>	<b>Mechanism of action</b>	<b>References</b>
Chrysin (CS)	<i>In vivo</i>	TNF- $\alpha$ , IL-17A and 22	115
Rhodomyrton	<i>In vivo</i>	Inhibits TNF- $\alpha$ , IL-17A	116
Rhododendrin	<i>In vivo</i>	IL-1,6,8,17,23 and TNF- $\alpha$	117
Amentoflavone	<i>In vivo</i>	Inhibits IL-17A, IL-22 and IL-23	118
Periplogenin	<i>In vivo</i>	Anti-necrotic agent	119

## 2.8 Preferred Plant

### 2.8.1 Passiflora

Passiflora is a genus, which has 500 species. It is the largest in the Passifloraceae family (120). It was widely distributed in new world and rarer in tropical Africa, Australia and Asia (121)



**Figure 2. *Passiflora caerulea*. Linn**

#### Scientific classification of *Passiflora caerulea* (121)

<b>Kingdom</b>	Plantae
<b>Order</b>	<i>Malpighiales</i>
<b>Family</b>	<i>Passifloraceae</i>
<b>Genus</b>	<i>Passiflora</i>
<b>Species</b>	<i>P.caerulea</i>
<b>Binomial name</b>	<i>Passiflora caerulea</i> . Linn

**Chemical Constituents:** It contains chrysin, cyanogenic glycoside sulphate tetraphyllin B-4 sulphate and epitetraphyllin B-4 sulphate (121,123)

**Drug name**

CS

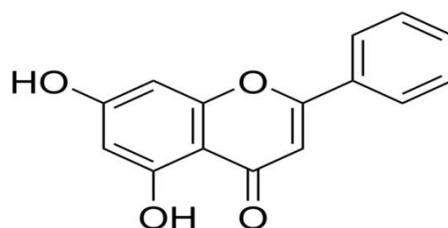
**IUPAC name**

5,7- di-hydroxy 2-phenylchromen-4-one

**Synonym**

Chrysin, 480-40-0, 5,7-dihydroxyflavone/5,7 Dihydroxy-2-phenyl-4H-chromen-4-one

### Chemical structure



### Molecular weight

254.24 g/mol

### Molecular formula

C<sub>15</sub>H<sub>10</sub>O<sub>4</sub>

### Physical appearance

Yellow powder

### Melting point

285.5°C

### Boiling point

491.91 °C

### Log P

3.52

### Solubility

"CS" is practically insoluble in water (84 mg/L). Soluble in sodium hydroxide and methanol, slightly soluble in alcohol, chloroform, ether and acetonitrile (124).

### Mechanism of action

CS reduces "IL-17A", "TNF"- $\alpha$ , "IL-22" induced "CCL20" and "AMP" release from epidermal keratinocytes at 30 $\mu$ M dose (121). It downregulates the "COX-2", "NF-kB" proteins, "PCNA" expression and furthermore it maintained cellular homeostasis, anti

metastatic activity by regulating MMP-10 and epithelial-mesenchymal transition (122,123). Additionally, CS reduces cell proliferation, inflammation and induced apoptosis for inhibition of cell growth (121-125). CS shows anti proliferative effect by reducing the levels of P21; P53; P63; P73; Rb; Cyclin D1, Cyclin E; Cyclin: B1, PCNA, CDK-2-4-6-8. Apoptosis effect by reducing the levels of cytochrome C, Bax;BCl2;Bad;XIAP;CASPASE3-8-9, $\beta$ -arrestin, survivin, mcl and PARP. It exhibits anti-inflammatory effect by acting on P38 MAPK, JNK, INFs, ILS, HO-L; P13K, AKT, TNFS, GSK- $\beta$ , IKK- $\beta$ , ICAM-I, VCAM-L and iNOS. It acts on enzymes like COX2, SOD, MDA, GPX, GSH and CAT there by it produce the antioxidant property (121-125).

CS has the antipsoriatic activity on imq induced psoriasis like mice at the dose level of 3  $\mu$ M to 50  $\mu$ M. Although its pharmacological activity is dose dependent due its cytotoxic effect at 50  $\mu$ M or More. A dose below the 50 $\mu$ M of CS was selected for its further exploration in dosage form. (115)

### **Category**

Anticancer agent (126) Anti-inflammatory agent (127) anti-proliferative agent (128), proapoptotic agent(129), anti angiogenic agent, anti-diabetic agent, anti-metastatic agent (130), cardio protective (131), neuroprotective, (132), antibacterial (133),immunomodulator (134), antioxidant, antidepressant (135) antiviral (136) and hepatoprotective agent (137)

### **2.9 Challenges with Chrysin (51)**

- Insoluble in water
- Poorly absorbed when given orally
- Rapidly metabolized (Conjugation, glucuronidation and sulfation) and eliminated
- Bioavailability is less (0.003-0.02%)
- Undergoes first pass effect

## 2.10 Human microbiome

Human microbiome is the emerging concept relating to the commensal microbial cells harboured by each individual, primarily on skin and in the gut. The study of the human microbiome began with Antonie van Leewenhoek (138) as early as 1680s. He correlated his oral and faecal microbiota between these habits. He noticed the significant difference in between samples from individuals in both of these locations in healthy and disease state. Thus research about microbes at various sites of body in the healthy as well as disease condition was as old as microbiology itself (138)

The advances in next generation sequencing (NGS), 16S rRNA, DNA gene sequencing, internal transcribed spacer 1 (ITS1) region and whole genome metagenomic shotgun sequencing techniques reveals the composition of the skin, GIT microbiome and its implications for skin health and disorders(139) The normal composition of microbiota of the skin and GIT is depends upon the age, mode of delivery, maternal microbiota, baby feeding strategy, genetics, and environmental variables such as dietary choice (140)

The largest organ of the body is skin (1.8 m<sup>2</sup>),(141) which is made up of outer epidermis, inner dermis and hypodermis. (141,142) The skin gives protection from external environment, UV, mechanical pressure and microbes. It regulates the water, electrolyte balance, body temperature and maintains homeostasis of body. It eliminates waste material in the form of sweat and synthesizes the Vitamin D3. The skin acts like ecosystem to wide range of microorganism. They help in maintaining physical barrier function by preventing pathogen penetration and invasion (141,142).A unique own microbiota was associated with cutaneous invaginations, appendages, sebaceous glands, sweat glands and hair follicle. To the hair follicle sebaceous glands are connected which forms the pilosebaceous unit, and secretes the lipid rich substance i.e sebum. Sebum provides lubrication and antibacterial shield to the skin. Sebaceous glands are relatively maintains the anoxic conditions which was suitable for growth of facultative anaerobes such as *Propionobacterium acne* (*P.acnes*) (143). *P.acne* is a common skin commensal bacterium, as per full genome

sequencing technique, it degrades the skin lipid of sebum. It also hydrolyses the triglycerides present in sebum and releasing free fatty acid present on the skin. The free fatty acids contribute the acidic pH of skin, which ultimately inhibits the pyrogens such as *Staphylococcus aureus* and *Streptococcus pyogenes*. They also favour the growth of the coagulase negative *Staphylococci* and *Corynebacteria* (144-145)

The sweat glands are of two types. One is eccrine sweat gland and other one is apocrine sweat gland. Eccrine sweat glands distributed throughout the skin which secrete the sweat composed of salt and electrolytes. It helps in the acidification of skin ultimately kills the pathogen. Furthermore, eccrine sweat glands produce the cathelicidin and  $\beta$ -defensins like antimicrobial peptides (AMP) result in inhibition of pathogens. (145)

Skin commensal microbes have recently been demonstrated that they modulate the host immune system that ultimately maintains the skin homeostasis. The composition of microbiota on skin, rely on the site of the body which can be categorized as dry, moist and oily sites (sebaceous site). The list of the composition of the microbiota at various sites were tabulated in the Table 5 (146). The list of skin commensals have described in the Table 6 along with their role. They inhibit pathogens by secretion of various antimicrobial peptides (147). The AMP include short peptides, proteins, lipids and small molecular chemical compounds (147)

**Table 5.** The list of the composition of the microbiota at various sites.

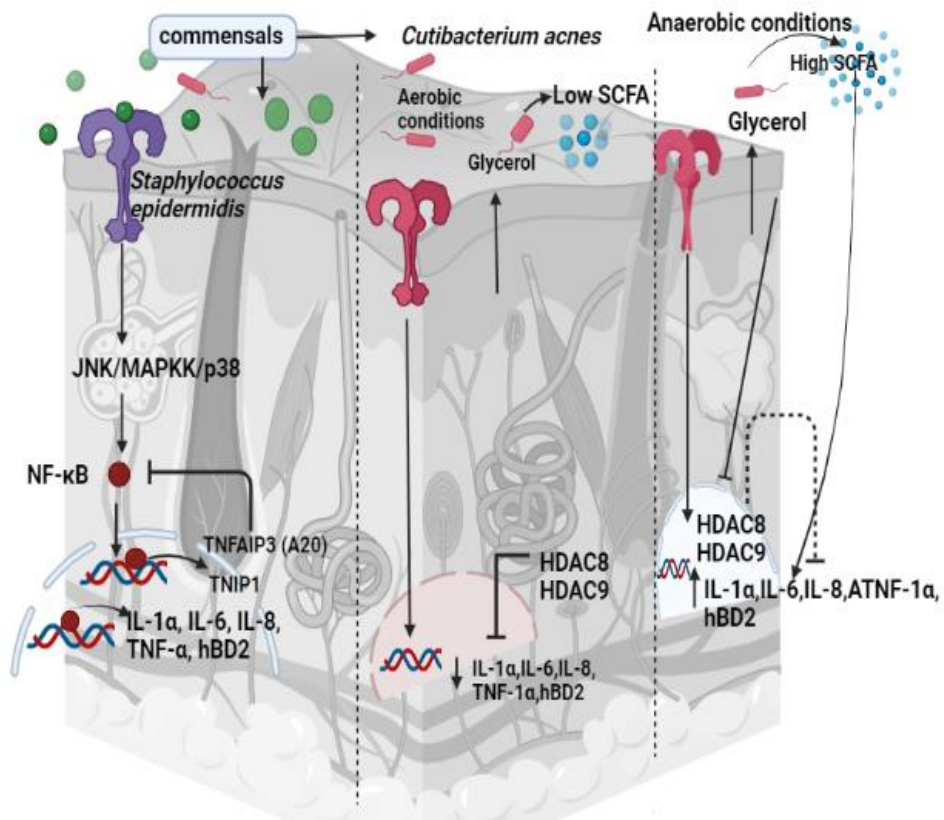
	<b>Bacteria</b>	<b>Eukarya</b>	<b>Virus</b>
D R Y	<i>Staphylococcus capitis</i>	<i>Parachlorella kessleri</i>	<i>Molluscum contagiosum virus</i>
	<i>Veillonella parvula</i>	<i>Epidermophyton floccosum</i>	<i>Stenotrophomonas phage</i>
	<i>Staphylococcus epidermidis</i>	<i>Zymoseptoria tritici</i>	<i>Human papilloma virus (β)</i>
	<i>Propionobacterium acnes</i>	<i>Nannizzia nana</i>	<i>Actinomyces phage</i>
	<i>Corynebacterium tuberculostearicum</i>	<i>Malassezia globosa</i>	<i>Streptococcus phage</i>
	<i>Streptococcus oralis</i>	<i>Candida parapsilosis</i>	<i>Polyomavirus HPyV7</i>
	<i>Streptococcus pseudopneumoniae</i>	<i>Malassezia sympodialis</i>	<i>Stenotrophomonas phage</i>
	<i>Streptococcus sanguinis</i>	<i>Malassezia restricta</i>	<i>Simian virus</i>
	<i>Micrococcus luteus</i>	<i>Pyramimonas parkeae</i>	<i>Acheta domestica densovirus</i>
	<i>Streptococcus mitis</i>	<i>Aspergillus tubingensis</i>	<i>Merkel cell polyomavirus</i>
M O I S T	<i>Corynebacterium simulans</i>	<i>Malassezia restricta</i>	<i>Polyomavirus HPyV6</i>
	<i>Staphylococcus epidermidis</i>	<i>Aspergillus tubingensis</i>	<i>Staphylococcus phage</i>
	<i>Corynebacterium tuberculostearicum</i>	<i>Zymoseptoria tritici</i>	<i>Acheta domestica densovirus</i>
	<i>Corynebacterium afermentans</i>	<i>Malassezia globosa</i>	<i>Molluscum contagiosum virus</i>
	<i>Staphylococcus capitis</i>	<i>Tilletia walkeri</i>	<i>Propionobacterium phage</i>
	<i>Propionobacterium acnes</i>	<i>Nephroselmis olivacea</i>	<i>Merkel cell polyomavirus</i>
	<i>Corynebacterium fastidiosum</i>	<i>Pramimonas parkeae</i>	<i>Polyomavirus HPyV6</i>
	<i>Enhydrobacter aerosaccus</i>	<i>Malassezia sympodialis</i>	<i>Actinomyces phage</i>
	<i>Micrococcus luteus</i>	<i>Cyanophora paradoxa</i>	<i>Enterobacteria phage</i>
	<i>Staphylococcus hominis</i>	<i>Parachlorella kessleri</i>	<i>Human papillomavirus(γ)</i>
S E B A C E O U S	<i>Propionobacterium acnes</i>	<i>Malassezia sympodialis</i>	<i>Acheta domestica densovirus</i>
	<i>Corynebacterium aurimucosum</i>	<i>Aureoumbra lanunensis</i>	<i>Human papillomavirus(β)</i>
	<i>Staphylococcus hominis</i>	<i>Pramimonas parkeae</i>	<i>Enterobacteria phage</i>
	<i>Staphylococcus capitis</i>	<i>Gracilaria tenuistipitata</i>	<i>Human papillomavirus(γ)</i>
	<i>Corynebacterium simulans</i>	<i>Luecocytozoon majoris</i>	<i>Merkel cell polyomavirus</i>
	<i>Streptococcus mitis</i>	<i>Tilletia walkeri</i>	<i>Gamma papillomavirus HPV127</i>
	<i>Staphylococcus epidermidis</i>	<i>Parachlorella kessleri</i>	<i>Propionobacterium phage</i>
	<i>Corynebacterium kroppenstedtii</i>	<i>Pycnococcus provasolii</i>	<i>Polyomavirus HPyV6</i>
	<i>Corynebacterium amycolatum</i>	<i>Malassezia restricta</i>	<i>Molluscum contagiosum virus</i>
<i>Corynebacterium tuberculostearicum</i>	<i>Malassezia globosa</i>	<i>Staphylococcus phase</i>	

**Table 6.** A List of commensal of skin and their role

<b>Name of bacteria</b>	<b>Role</b>	<b>References</b>
<i>Staphylococcus Simulans</i>	Synthesize the Auto inducing peptides, which inhibits the colonization of <i>S.aureus</i> Inhibits the methicillin resistant <i>S.aureus</i>	(148)
<i>Malassezia globosa</i>	Secrets protease, limits the growth of <i>S.aureus</i>	(149)
<i>S.hominis</i>	It secretes AMP, which inhibits the growth of <i>S.aureus</i>	(150)
<i>Staphylococcus caprae</i>	It produce the auto inducing peptides, there by <i>S.aureus</i> Agr	(151)
<i>S.lugdunensis</i>	Secreted the thiazoline containing cyclic pepetide antibiotic called lugdunin	(152)
<i>Staphylococcus epidermidis</i>	Serine protease inhibits the <i>S.aureus</i> colonization and biofilm	(153)
Coagulase negative <i>Staphylococcus</i> (CoNS) species	Production of antimicrobial peptides (AMP), lantibiotics for the inhibition of <i>Staphylococcus aureus</i>	(154)
<i>Corynebacterium spp.</i> ,	Eradicates <i>S.aureus</i>	(155)
<i>S.epidermidis</i>	Increased the expression of $\beta$ defensin and promotes the AMP from keratinocytes Expresses phenol soluble modulins, Which ultimately kills <i>Streptococcus</i> Pyogenesis (GAS) and <i>S.aureus</i> It also produces the 6-N-hydroxyl amino purine (6-HAP), which has inhibitory effect against GAS and inhibits skin neoplasm It also releases serine proteases cleared nasal <i>S. aureus</i>	(156,159)

Commensal microbes communicate with immune system by the induction of the quorum sensing property or accessory sensing molecule, For example skin commensal *S.epidermidis* upon sensed by keratinocytes of Toll like receptor 2 Increased the expression of  $\beta$ -defensins 2 and 3 Block the NF-kB inhibition, induced by the *S.aureus* pathogen





**Figure 3. Mechanism of skin homeostasis by skin commensals (159)**

Skin commensal plays the major role in the maintenance of the immune system homeostasis of skin by releasing the various the short chain fatty acids and antimicrobial peptides. According to recent research, to establish the skin immune system homeostasis, skin commensal activate the negative regulators of TLR signalling in cell membrane. A diagrammatic representation of mechanisms, how skin commensal maintains the homeostasis of the skin was shown in **Figure 3** For example "*Staphylococcus epidermidis*" and *Cutibacterium acnes* induces the "TNFAIP3" (tumour necrosis factor alpha induced protein 3) and "TNIP1" (TNFAIP3 interacting protein1) proteins, which impact the, progressive expression of cytokines (159). In that scenario, pilosebaceous unit of *Cutibacterium acnes*, secrete the short chain fatty acids (SCFA) by utilizing the glycerol as metabolizing agent under aerobic conditions. The produced SCFA which include propionate and valerate were detected by the keratinocytes and sebocytes which ultimately inhibits the HDAC8 (Histone deacetylase) and HDAC9, it leads to blocking of the secretions of cytokines.

In addition to that, SCFA have the capacity to regulate the expression of cytokines, via stimulation of the free fatty acid receptor. A good skin is the representation of proper functioning of skin microbiome. However the species composition, abundance and distribution of the microbiome depends upon the "external (hygiene, sunlight, beauty routine, climate, physical activity, exposure with chemicals, and availability of nutrients for microorganism) and internal (age, immunity, genetics, stress, sleep, gender, hormones, and hormones) factors (160)

The microbiota of the skin region differs in conditions like moisture, P<sup>H</sup>, temperature, sebum content, ultraviolet exposure and topographical conditions. It also depends upon the environmental, life style and location of working individual. The highest concentration of "*Staphylococcus* and *Corynebacterium* species", were located in wet areas such as elbows and soles of the feet (161). The combination of the "*Rhodotorula spp.*, "*Aspergillus spp.*," "*Epicoccum spp.*, and "*Cryptococcus spp.*," were abundant at foot sites and *Malassezia* were at core body and the feet region.

### **2.11 Dysbiosis of skin in psoriasis**

A recent study revealed that the heterogeneous diversity of the skin microbiome in psoriasis skin was observed, when compared with healthy skin. In addition to that antimicrobial peptides, LL-37,  $\beta$ - defensin and psoriasin were abundant with constant presence on the psoriasis lesions, leads to the hyper proliferation of keratinocytes. Furthermore, the constant of antimicrobials undermine the healthy skin microbiome. It could be due to the life style change, diet, excessive bathing, and cosmetic application which progress the skin irritation, dryness, altered barrier function (162) Assarsson *et al.*, in 2018 analysed the dysbiosis of skin in plaque type psoriasis patients by Targeting 16 S rRNA gene V3–V4 for Mi sequencing, V13.516 S rRNA sequencing (V1–V2) and this study reports as the "*Firmicutes*" and "*Staphylococcus*" were reduced in the psoriasis skin (162-163). Langan *et al.* in 2018 observed the decreased diversity of *Actinobacteria*, *Anaerococcus* and *Propionibacterium* in psoriatic skin analysed by the RDP traditional culture combined with mass-spectrometry (MALDI-TOF) (164) Quan *et al.* in 2020 were demonstrated the

decreased quantity of *Cutibacterium*, *Streptococcus* and *Deinococcus* with the help of targeting 16 S rRNA gene analysis technique in V3–V4 region (165).

Fyhrquist *et al.* in 2019 applied the metagenomic shotgun sequencing method for the analysis of dysbiosis of psoriasis skin it has been concluded as the decreased diversity of *P. Acnes*, *Lactobacilli*, *Burkholderia* spp. and *Corynebacterium* spp., (166)

Alekeyenko *et al.* and Gao *et al.* found less bacterial diversity in psoriatic skin, than the healthy skin, which was listed in the Table 7 (167)

**Table 7.** Comparison of microbiome in psoriasis and healthy skin

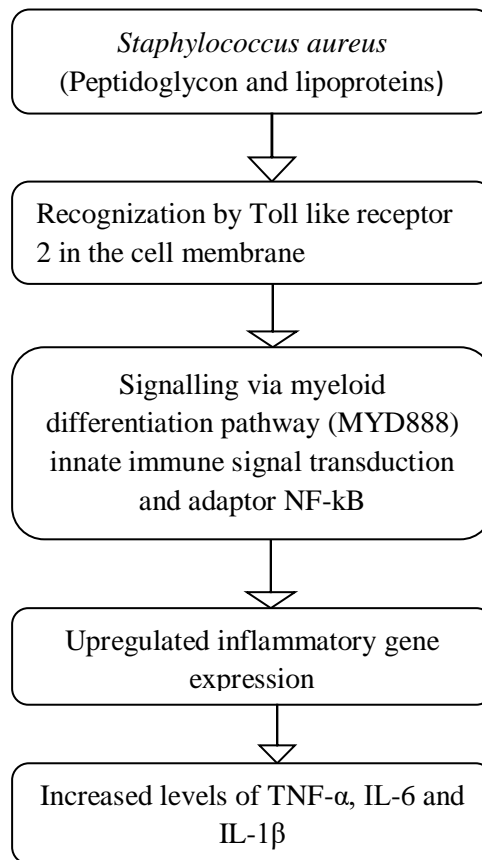
Psoriasis skin	Healthy skin	References
Increased levels of <i>Corynebacterium</i> , <i>Propionibacterium</i> , <i>Staphylococcus</i> , <i>Streptococcus</i>	Increased Proteobacteria	167
Decreased <i>Malassezia</i>	Increased <i>Malassezia</i>	168
<i>Streptococcus</i> > <i>Staphylococcus</i> , Proteobacteria and Propionibacteria	Increased Actinobacteria	169
Increased diversity of firmicutes Actinobacteria	Increased Proteobacteria Actinobacteria	170

Chang *et al.*, in 2018 conducted the sequence study by the 16rRNA at V1-V3 variable regions and analysed that there is a significant difference between the psoriatic associated and healthy skin microbiota. Their study displayed, relative abundance of the *Staphylococcus aureus* on lesional and non-lesional psoriatic skin. However "*Propionibacterium acnes*" and "*Staphylococcus epidermidis*" were underrepresented in psoriatic lesions when compared with healthy skin. They strongly concluded that murine model of *Staphylococcus aureus* (*S.aureus*) suggest that *S.aureus* triggers Th-17 type response, which ultimately leads to induction of "IL-22", "IL-17A", and "IL-17F" "inflammatory mediators" (171). Atefi N *et al.* in 2014 conducted the case report study of psoriatic patients, from which they were found that the *Staphylococcus aureus* level was 25 times higher in the serum when compared with normal healthy patients (172).

*S.aureus* is the gram positive bacteria it secretes many virulent factors (pore forming toxins, phenol soluble modulins, exfoliative toxins and supra antigen) and enzymes which causes dysregulation of immune system which ultimately leads to progression (auto reactive T-cells) of the chronic autoimmune system (173) The list of virulence factors and their activity in the damaging of the host cell are listed in the Table 8 (174) The released toxins, exacerbated the inflammatory responses by activation of various immune cells, which include macrophages, eosinophils, dendritic cells, mast cells, basophils, keratinocytes, helper T-cells, innate lymphoid cells and neutrophils. Ultimately the activated, immune cells secretes various cytokines and induce the responses of inflammation. *S.aureus* when breaches the skin, via tissue injury or pore forming activity. It recognized by the TLR2, and unregulated the gene expression by the NF-kB, which leads to the production of the inflammatory cytokines which includes "TNF- $\alpha$ ", "IL-6" and "IL-1 $\beta$ ". In the flow chart (Figure.4) form, the way of *S.aureus* cell components interacts with immune system was described (175,176)

**Table 8.** The list of virulence factors and their activity in the damaging of the host cell (173,176)

Virulence factor	Function
Pore forming toxins $\alpha$ -toxin, Panton-Valentine leukocidin, $\alpha$ , $\beta$ and $\gamma$ -heamolysis	Lysis of eukaryotic cell Altered permeability of cell membrane Heamolysin- $\alpha$ , forms the pore in the cell membrane, which causes the release of the potassium ion and also induces the production of the IL-1 $\beta$ ,IL-6,IL-8 Activation of caspase-1,3, 12 and generation of proinflammatory cytokines Induction of apoptosis and necroptosis $\beta$ and $\gamma$ -heamolysis
$\alpha$ -toxin	Causes cellular autophagy, activates receptor interacting serine/threonine protein kinase 3, caspase-8, casepase-12, necroptosis and apoptosis
Panton-Valentine leukocidin	It causes the leukocyte destruction, apoptosis and pyroptosis Cell lysis of the macrophages and neutrophils
Peptidoglycon cell membrane component of <i>S.aureus</i>	Activation of keratinocytes, langerhans, macrophages, CD+4T cells, Th1 cells, microglia and induces the release of the several inflammatory mediators which includes " IL-6", "TNF- $\alpha$ ", "IL-13", "IL-8", "IL-1", "IL-5", "IL-4" and 'autophagy".
Extracellular adhesion proteins of <i>S.aureus</i>	Acts on T-cells



**Figure 4.** Representation of *S.aureus* interaction with immune system (173,176)

## 2.12 Probiotics

WHO defined as probiotics are live microorganism, which when consumed in suitable amounts which confer a health effect on the host. Probiotics are safe and affordable to common peoples in contrast to the existing targeted treatments such as biological agents. Mostly human and animal origin microorganisms are utilized for the therapeutic effects.

*Bifidobacterium* and *lactobacillus* have several favourable effects, which depends on the selection of the probiotics. These can be selected on the basis of safety, functionality and technical usability (177). The some of the ideal characters of the probiotics and their functions are mentioned below.

### 2.13 Ideal characters of probiotics

- Must be safe for consumption and effective in providing health benefits
- Non pathogenic
- Anti biotic resistant
- Production of anti microbial agents (178,179)

### 2.14 Functions of probiotics (178,179)

- Acts as a immune-modulating agents
- Gives protection against "*Salmonella typhimurium*", "*Helicobacter pylori*", and "*Escherichia coli*" pathogenic bacteria
- Decreasing cholesterol levels in serum
- Used in Crohn's disease management
- Used to treat "ulcerative colitis" and "irritable bowel syndrome" (IBS)

A few of the important functions of probiotics are tabulated in the Table 9 (180, 181)

**Table 9.** Functions of Probiotics (180-181)

• Immune system activation which leads to production of various antibody, NK cells and T-regulatory cells
• Modulation of cytokine, NF-kB and AP-1 pathways.
• Remodelling of dendritic cell phenotype and function
• Increased protein phosphorylation caused the epithelial barrier protection followed by tight junction integrity
• Improved epithelial cell glycosylation
• Production of bacteriocins, peroxides which caused the anti-microbial effects on the pathogens
• Inhibition of pathogenic bacteria invasion
• Reduction of lactose production
• Targeting of tumour
• Intolerance
• Cholesterol reduction

Live probiotics have been exploited either to treat or prevent inflammatory conditions. Many probiotics have been shown to attenuate the inflammatory mediators, including IL-8 (182-183) Positive beneficial effects are showing towards probiotics for psoriasis treatment revealed by researcher's (184)

A list of probiotics used for psoriasis and their preclinical and clinical studies are discussed in Table 10. The mechanism of action of probiotics is as follows (185,186)

- Epithelial barrier protection
- Inhibition of pathogenic bacteria invasion
- Secretion of anti-microbial peptides
- Acts as an immunological modulators
- Stimulates IL-12, IFN- $\gamma$ , and IL-10

**Table 10. Preclinical and clinical evaluation of probiotics for psoriasis**

S.No	Probiotic	Route	Dosage	Mechanism	Duration	Evaluation	References
1.	<i>Bifidobacterium infantis</i> 35624	Oral	1 $\times$ 10 <sup>10</sup> CFU per sachet/day	Plasma CRP IL-6 and TNF- $\alpha$	8 weeks	Clinical	187
2.	<i>Lactobacillus pentosus</i> GMNL-77	Oral	5 $\times$ 10 <sup>8</sup> CFU/day	IL-23 IL-17A/F IL-22 6 and TNF- $\alpha$	6 days	Preclinical	188
3.	<i>Lactobacillus sakei</i> proBio-65 Extract (SEL001)	Topical (heat killed)	50 mg/cm <sup>2</sup> on back and 10 mg on the right ear	IL-19 IL-17A and IL-23	6 days	Preclinical	189

Numerous drug delivery systems have been developed for delivery of probiotics. These are conventional beads like tablets and capsules, non-conventional such as milk, cheese, yogurts, meats, creams, and chocolates, etc (190).

Recent studies demonstrated as the huge rise in use of both topical or oral probiotics for skin diseases. Although oral administration of probiotics has effectively modulating the intestinal microbe and also demonstrated to be efficacious in treating the topical skin conditions which include atopic dermatitis, acne, rosacea.

The probiotics application on the skin promoted modulation of the immune system, pathogens reduction and balance of skin microbiome. (191) They have been proved for effective in the treatment of inflammatory skin diseases. Several topical probiotics related patents utilized for the treatment of the different skin diseases were effective without the adverse effects were listed in the Table 11 (191,192)

**Table 11.** List of topical probiotics of patents for the management of the different skin diseases (192)

<b>Patent No.</b>	<b>Probiotics composition</b>	<b>Category</b>
WO2015/181534A1	<i>Lactobacillus rhamnosus</i>	For skin tissue and wound repair
FR2938429B1	<i>Lactobacillus</i> or <i>Bifidobacterium</i>	Restores natural scalp function, reduces irritation, itching and dandruff
CN111601583A	"Lysate of the genus bacillus"	management of seborrheic condition in particular bacterial growth and inflammation dependent on sebum



## 2.15 Selection of probiotic

### 2.15.1 "*Bifidobacterium infantis*"

"*Bifidobacterium*" is a genus of gram positive, non-motile, branched rod shaped anaerobic bacteria (193).



#### *Scientific classification of Bifidobacterium infantis (194-195)*

<b><i>Phylum</i></b>	<i>Actinobacteria</i>
<b><i>Class</i></b>	<i>Actinobacteria</i>
<b><i>Order</i></b>	<i>Bifidobacteriales</i>
<b><i>Family</i></b>	<i>Bifidobacteriaceae</i>
<b><i>Genus</i></b>	<i>Bifidobacterium</i>
<b><i>Species</i></b>	<i>B. longum</i>
<b>Binomial name</b>	<i>Bifidobacterium longum</i>

*B. infantis* was available in various food products such as sauerkraut, yogurt, cheese, salami and olives (194,195)

#### **Mechanism of action**

*B. infantis* inhibits the interleukin 6 and TNF- $\alpha$  (196)

#### **Category**

Regulate immune system, immune modulator, induce FOXP3 and IL-10 (197). It used in the treatment of ulcerative colitis, irritable bowel syndrome (198), chronic fatigue syndrome and psoriasis

Bifidobacterium species were able to produce the acetic acid (3.32 mg/mL) during fermentation(199,200). It has the bactericidal effect (*S. aureus*) and also most of the reports has been represented as that, it can be used for treatment of burns and superficial infections.

## **2.16 Novel drug delivery system for psoriasis**

Although herbal drugs are thought to be safe, easily available with affordable cost and having fewer side effects. However herbals acts on multiple sites, limited by less solubility and permeability which reduces the less absorption of drug through skin. In addition they have similar physicochemical problems as synthetic drugs such as insolubility, instability and poor bioavailability. These physicochemical and physiological obstacles can be overcome using a variety of nanotechnologies (201). It reduces the dosing frequency, improves bioavailability, minimizes drug degradation, and achieves a therapeutic window at a sustained level. These systems overcome the adverse effects of conventional drug delivery systems. To deliver the drug to the specific target site of the body, carrier-based delivery systems are used. These vehicles adhere to the skin effectively, maintain the sustained level of drug in the blood and prevent water loss from the skin. The use of various drug-delivery systems such as ethosomes, niosomes, solid lipid nanoparticles (SLNs), liposomes, polymeric nanoparticles, NLCs, and metallic nanoparticles for successful delivery of phyto-medicines to their respective targets were discussed below (201, 202, 203). In addition to that a list of the currently, developed and evaluated numerous nano phyto-pharmaceuticals are also given in **Table 12**

### **2.16.1 Lipid-based Nano-carriers**

Lipid-based nanocarriers are prepared with physiological lipids which are free from toxicity, biodegradable, enhance stability, and economical. These nanocarriers comprises SLN and NLCs

### **2.16.1.1 Capsaicin (CAP) loaded SLNs and NLCs**

Agrawal *et al.* in 2015 developed the lipid nanoparticles containing CAP. The CAP loaded NLCs was compared with the CAP loaded SLNs, NLCs exhibited higher drug loading, lower size with higher drug entrapment efficiency, higher skin permeation and no skin irritation. Depending upon the desired drug permeation profile and deposition of drug at target site NLCs are the better option for skin delivery. This report concluded that the NLCs are more suitable for topical application (204,340).

### **2.16.1.2 Mometasone furoate (MF) NLCs**

MF is a prodrug, corticosteroid that is mainly used topically to treat psoriasis and eczema. It inhibits the synthesis of cytokines including IL-1, IL-6, and TNF- $\alpha$ . The conventional drug delivery system of *Mometasone furoate* has number of issues, which including low drug uptake due to the stratum corneum, systemic absorption, skin burning, swelling of hair follicles and may cause skin atrophy if used for an extended time. NLCs have the ability to improve skin retention at the target site, it also lowering the risk of local and systemic risk linked with topical corticosteroids. Kaur *et al.* in 2018 loaded MF in to the NLCs, incorporated in to the carbopol 940 based hydrogel and evaluated the activity of optimized formulation. The drug permeation study of NLCs based gel has shown the prolonged drug release when compared with the marketed product. *In vivo* study of MF-loaded NLCs on IMQ induced psoriatic skin mouse reduced the psoriatic lesions. It has been concluded as the use of NLCs based drug delivery system on topical application could reduced the side effects and it can be inferred that formulas based on NLCs are efficient (205,340)

### **2.17 Capsaicin (CAP) loaded vesicular system**

Gupta *et al.* in 2016 prepared and evaluated the CAP liposomes, niosomes and emulsions for localized and controlled topical drug delivery. The vesicular system incorporated in to the gel form. The comparative study of percentage drug release, degree of entrapment, skin permeation study, amount of drug in the skin and draize test of 3 vesicular systems were evaluated. The skin retention study of CAP loaded emul-gel formulation showed the more accumulated drug in the skin. It has concluded that the emul-gel could be promising dosage form for delivering CAP to the skin for successful psoriasis treatment (206,340)

### **2.18 Babchi oil microemulsion gel**

Ali *et al.* in 2008 investigated the oil contained in *Psoraleacorylifolia*, known as *babchi oil*, prepared as a micro-emulsion gel for psoriasis treatment.(207). An optimized microemulsion-based gel formulation increased *babchi oil* penetration in the skin and has anti-inflammatory activity in a foot pad oedema model (208,340)

### **2.19 Turmeric microemulgel**

Sarafian *et al.*, in 2015 formulated the microemulgel and performed 3-week clinical trials. It was given topically for plaque psoriasis patients for the identification of its safety and efficacy. The Dermatology Life Quality Index (DLQI) Questionnaire and Psoriasis area & severity index (PASI) was used to evaluate the microemulgel treated patients with the untreated population. It has been reported that PASI score in microemulgel treated patients improved ( $P < 0.05$ ) when compared with untreated population (209,340)

### **2.20 Thymoquinone(TQ) loaded ethosomes**

Negi *et al.* in 2019 has developed the TQ loaded ethosomes to overcome the problems of TQ, such as hydrophobicity, low aqueous solubility and photosensitivity. The formulated TQ ethosomes are loaded into hydrogel.

Furthermore hydrogel was evaluated for the antipsoriatic activity on Swiss albino mouse by employing tail model. The percentage antipsoriatic property of TQ loaded ethosomes, plain TQ, NS extract, marketed formulations was compared with each other in which TQ-loaded ethosomal gel has shown the better results. As a result of this research, it was concluded that TQ in the form of ethosomal hydrogel has shown the better results upon topical application (210,340)

## **2.21 Polymer based nano-carriers**

Polymeric Nanoparticles are solid structures that can either encapsulate or absorb bioactive molecules. It has shown great promise in the delivery of drugs to specific locations for the treatment of a variety of diseases. They consist of nano-spheres, nano-capsules, and dendrimers. Polymeric nanoparticles protect the drugs from oxidation, which also improves the solubility and bioavailability. Polymeric nanoparticles were created using a variety of biodegradable polymers. Polylactic acid, poly (lactide-co-glycolic acid) (PLGA), polyglycolic acid, and poly(cyanoacrylate) are used to create the hydrophobic zone (caprolactone). The hydrophilic element of PEG has been widely used (210,340)

### **2.21.1 PLGA-Curcumin nanoparticles**

*Curcumin* (CUR) loaded PLGA nanoparticles (NPs) were developed and administered topically on IMQ triggered psoriasis-like mouse model. It has increased the aqueous solubility and chemical stability of CUR. The CUR bioactivity was greatly improved by encapsulating lipophilic CUR in PLGA NPs in the mouse skin. The PLGA NPs system improved the drug dispersion, enhanced the drug penetration, more drug deposited in the skin and circulation along with sustained release (210,340)

### **2.21.2 Curcumin polymeric nanoparticles based hydrogel**

Mao *et al.* in 2017 formulated the CUR loaded Polymeric "(RRR- $\alpha$ -tocopheryl succinate grafted e-polylysine conjugate (VES-g-e-PLL) nanoparticles and incorporated in to the silk fibroin hydrogel for topical application". They evaluated the activity of CUR loaded nanoparticles and the CUR containing nanoparticles gel on IMQ-induced psoriatic mice model. It revealed that CUR-NPs-gel has a greater

effective skin-permeating effect and efficient anti-keratinization mechanism than CUR-NPs. As a result, inhibited cytokines release (IL-6, NFkB and TNF- $\alpha$ ) more effectively (212). It has concluded that the permeable nanoparticle-gel formulation could be used to incorporate lipophilic antipsoriatic drugs for topical administration (213)

**2.22 Metallic based Nano-Carriers:** These are inorganic in nature and biocompatible, showing good stability, for a long period. They have the potential to target the site and exhibit cellular uptake. Mainly utilized for transdermal delivery but may cause toxic effects. Predominately they comprise silver and gold nanoparticles. The gold nanoparticles have a size, in the range of 0.8 -200nm. The polyphenolic extract of *Cornusmus* loaded gold and silver nanoparticles were formulated and evaluated by the Crisan D *et al.* in 2018. It has shown the anti inflammatory effects in bonemarrow derived murine macrophages by reducing the Nitrous oxide, TNF- $\alpha$  and IL-12 (214,340)

**2.22.1 Hypoxis hemerocallidea extract and hypoxoside gold nanoparticles (AuNPS):** Elbagory *et al.* in 2019 synthesised the AuNPs from the extract of *H. hemerocallidea* and *hypoxoside* secondary metabolite. They conducted the immunomodulatory effect study of the aqueous extract of *H. hemerocallidea*, *hypoxoside*, AuNPs derived from the extract and *hypoxoside* using a solid phase sandwich ELISA technique. (215). The finding showed that "H.hemerocallidea extract, hypoxoside, and their corresponding AuNPs may decreased cytokine levels in macrophages, but only *hypoxoside*-derived AuNPs can supress the proinflammatory cytokine responses in NK cells" (216,340)

**Table 12. Nano phyto-pharmaceuticals for psoriasis**

<b>Phyto-drugs</b>	<b>Formulation</b>	<b>Mechanism of action</b>	<b>Evaluation</b>	<b>Results</b>	<b>References</b>
Curcumin	Nanohydrogel	Inhibits the expression of cyclin E", "TNF- $\alpha$ , IL-1 $\beta$ , ,IL-6 "NF-KB and MAPK pathways	<i>In vivo</i> (IMQ)	Nanohydrogel enhanced the water solubility of Curcumin, prevent the degradation of Curcumin and improved the penetration into the psoriatic skin	(213,217)
Mangiferin	Glycethosomes	It reduced the oxidation and inflammation	<i>In vitro or In vivo</i> (TPA)	Improved Mangiferin retention in the epidermis, reduced the oedema and inflammation	( 218,340)
Berberine oleate	Liquid crystalline nanoparticulates	Anti-inflammatory actions, Inhibition of cyclooxygenase lipooxygenase, cytokines release TNF- $\alpha$ , IL-17A, IL-23	<i>In vitro, Ex vivo and In vivo</i> (IMQ)	Increased skin penetration, skin deposition and Improved <i>in vivo</i> efficacy with inhibition of cytokines release	(219,220)
Curcumin	Hyaluronan-Modified Ethosomes	Lowers TNF- $\alpha$ , IL-17A, IL-17F, IL-22 and IL-1 $\beta$	<i>In vitro</i> HaCaT or <i>In vivo</i> (IMQ)	Improved skin permeability and <i>In vivo</i> skin retention	(221,340)
Celastrol	Niosomes	Inhibited the IFN- $\alpha$ , IL-17, IL-23, and IL-22 release	<i>In vitro</i> or <i>In vivo</i> (IMQ)	Enhanced <i>in vitro</i> permeation(465.3 $\pm$ 84.1ng/cm <sup>2</sup> ) and alleviated scaling and erythematic lesions on mice skin.	(222,340)

Epigallocatechin-3-Gallate	Chitosan-based polymeric nanoparticle	Reduced the skin thickness, erythema, scales, mastcells, proliferation, neutrophils, macrophages, CD4 T cells and angiogenesis	<i>In vitro/ In vivo</i> (IMQ)	Improve bioavailability and stability	(223,340)
Psoralen	Liposomes	Reduced the IL-22, TNF- $\alpha$ and IL-17	<i>Ex vivo/ In vitro/ In vivo</i> (IMQ)	Enhanced skin permeation and skin deposition. It showed safety and efficacy	(224,340)
Berberisarinata Extract47	Transfersomes-gel	Anti-inflammatory action and anti-psoriatic effect	<i>In vitro/In vivo</i> on IMQ	Avoids first-pass effect, enhanced in vitro release, improved anti-inflammatory activity	(225,340)
Ammonium Glycyrrhizinate	Niosomes	Reduced the inflammation, pain and oedema	<i>In vitro/In vivo</i>	Increased anti-inflammatory and anti-nociceptive responses	(226,340)
Psoralen	Ethosomes	Reduced the IL-17, TNF- $\alpha$ , and IL-22	<i>In vitro/In vivo</i> skin micro-dialysis	Skin deposition (6.56 folds) It reduced the toxicity and improved the efficacy	(227,340)



### **2.23 Nano structured lipid carriers (NLCs)**

A thorough analysis of various dosage forms in the management of psoriasis, lipid-based drug delivery could be suitable for the psoriatic skin. Mohd nordin *et al.* in 2021 review the lipid based nanoparticles for psoriasis treatment and concluded that the lipid carriers are the standard carrier system for topical administration due to its amphiphilic nature of the skin. (228). M.Elmoawfy and M.M. Al-Sanea in 2021 reviewed about NLCs and defined as the, binary lipid based carriers containing mixture of solid lipid (SLs) and liquid lipid (LLs). It allows the entrapment of the lipophilic drug and convenient for the dermal and transdermal drug delivery system. The study concluded as the NLCs hydrate the skin and mix with skin lipids (229). NLCs offer a unique solution to eliminate issues related with psoriasis i.e permeability of drugs through the skin. In addition they hydrate the skin by forming occlusive layer, and reducing the transepithelial water loss. (229,340)

#### **Definition**

NLCs are defined as the nano-particulate carrier system which are made up of solid lipids, liquid lipids, emulsifying agents and water. The mixture of solid lipids and liquid lipids leads to the formation of highly disorder matrix imperfection in carrier due to which higher amount of drug can be load. It also produces the sustained release of the drug from it (230). It has gained much more importance in the pharmaceutical development for several delivery routes. It has pharmaceutical applications in the management of various diseases which include hyper-tension, diabetes, cancer, hyper lipidemia, hepatic, alopecia, epilepsy and fungal diseases etc. NLCs can avoid the problems such as polymorphism, stability and loading efficiency (231,340 )

There are the three different types of NLCs, those are

1. The imperfect type

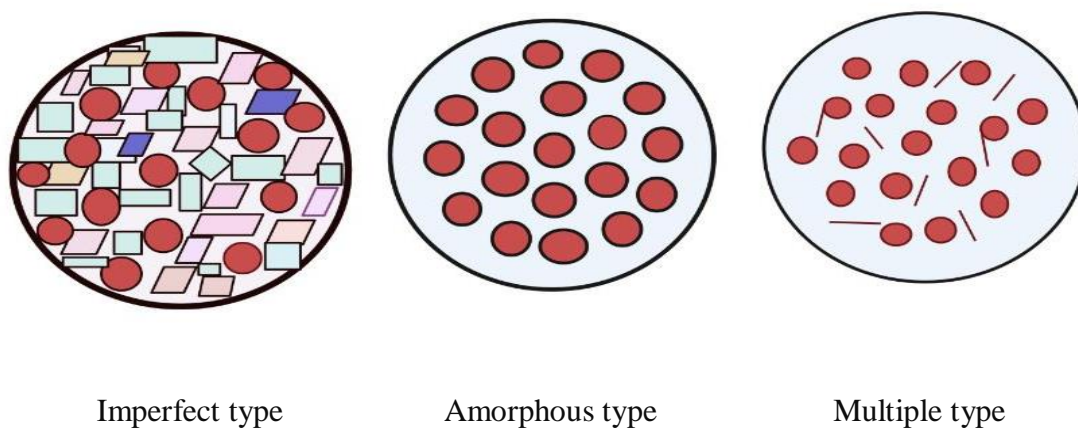
It has the solid matrix with imperfectly structure, with high drug loading capacity.

2. The amorphous type

It has the high amorphous matrix formation with less solid matrix, which showing the moderate drug loading.

### 3. The multiple type

It has multiple oil in fat in water type. All types of NLCs were shown in Figure 5



**Figure 5.** Types of NLCs

The type of formation of NLCs depends upon the type of method development and lipids used. (229-231)

#### **2.24 Advantages of the NLCs**

- NLCs improves bioavailability of highly lipophilic drugs with poor aqueous solubility
- Avoids the first pass effect
- The disturbed matrices type of structure in the NLCs provides the high % DL and % EE which ultimately sustained the release of drug from it (232)
- NLCs are physically stable
- Feasible and easy scale up process
- Increased skin occlusion effect
- To ensure the formation of small particle size carriers which aids the penetration of the drug

- The lipids utilized in the formulation development are safe, biocompatible and have regulatory acceptable range (Jaiswal *et al.*, 2016)

NLCs are considered as the smarter, latest generation lipid nanoparticles. They overcome the drawbacks of conventional topical therapy related to psoriasis treatment, such as pigmentation of skin, pain, poor drug diffusion and tingling effect on normal and diseased skin (230). NLCs reduced systemic toxicity, necrotizing effects of drugs and minimize the doses and minimize the doses. NLCs offer effective drug targeting, skin retention property and sustained drug release feature that might be suitable in successful management of psoriasis. NLCs could be potential, safe and biocompatible nano carriers for psoriasis as for as topical application is concerned. Furthermore, these NLCs can be converted into solid products upon lyophilisation or spray drying and further converted into tablets, pellets, capsules, and powder for reconstitution (230-234)

## 2.25 Methods for preparations of NLCs

There are many methods or techniques available for the development of the NLCs which are classified (Figure 6) based on the energy requirement for their development

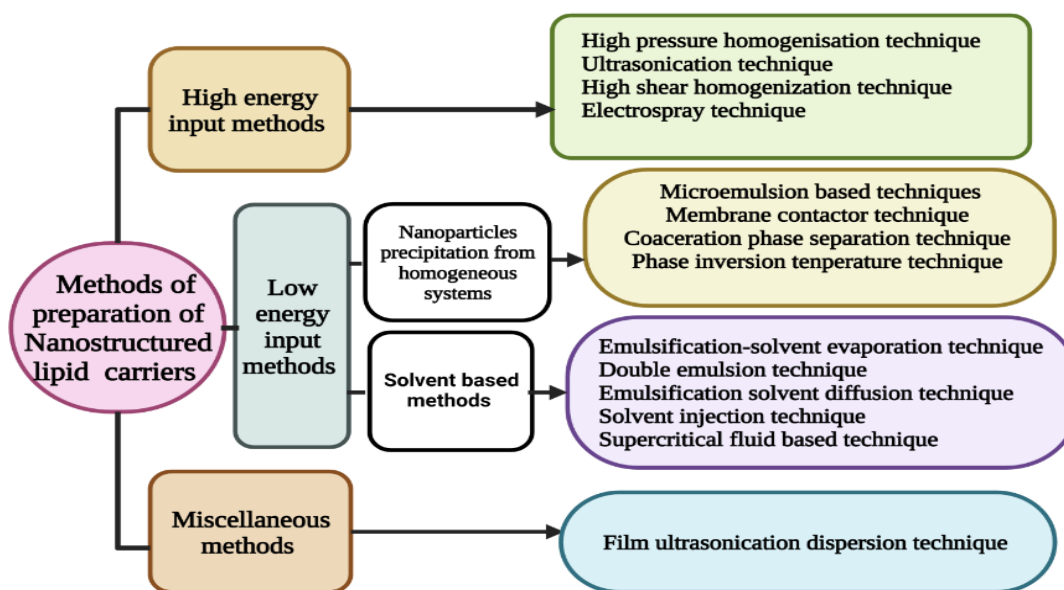


Figure 6. Classification of methods for the preparations of NLCs

However many preparations techniques available for the fabrication of NLCs, the selection of suitable method is dependent on the merits and demerits of it. The advantages and disadvantages of all the techniques were shown in the **Table 13**

**Table 13. Advantages and disadvantages of all the techniques**

Technique	Principle	Advantages	Disadvantages	References
"Hot high pressure homogenization technique"	Turbulent eddies induced "high cavitation forces"	<ul style="list-style-type: none"> <li>• Simple scale up on lab and industrial</li> <li>• Economical</li> <li>• Universally accepted method</li> <li>• Replicability of results</li> </ul>	<ul style="list-style-type: none"> <li>• High energy essential</li> <li>• Not suitable for thermo labile and hydrophilic drugs</li> <li>• High poly dispersity index</li> <li>• Ineffective homogenization during high temperature</li> <li>• Lipid modification during melting</li> </ul>	(230-238)
Cold pressure homogenization method	Turbulent eddies induced high cavitation forces	<ul style="list-style-type: none"> <li>• Suitable for thermo sensitive drugs</li> <li>• Higher drug loading can be achieved for hydrophilic drugs</li> <li>• Simple scale up process</li> <li>• No lipid modification during meting</li> </ul>	<ul style="list-style-type: none"> <li>• High energy needed</li> <li>• High Poly dispersity index</li> <li>• Long term storage may cause expulsion of drug</li> </ul>	(230-238)
High shear homogenization technique	Shearing between the stator and rotor	<ul style="list-style-type: none"> <li>• Energy dependent process</li> <li>• Feasible</li> <li>• Economical</li> </ul>	<ul style="list-style-type: none"> <li>• Physical unstable</li> <li>• Lesser encapsulation efficiency</li> <li>• Metal contamination</li> </ul>	(230-238)
Ultrasonication method	Higher cavitation forces	<ul style="list-style-type: none"> <li>• Feasible to scale up</li> <li>• Low shearing</li> <li>• Better process control</li> </ul>		

Electro spray technique	Electrical field solidifies the sprayed lipids	<ul style="list-style-type: none"> <li>Narrow particle distribution</li> <li>No particle aggregation</li> <li>Feasible</li> </ul>	<ul style="list-style-type: none"> <li>Destabilization of drug due to higher exposure of electric field</li> </ul>	(230-238)
Micro emulsion based technique	Rapid solidification of micro emulsion crystallization method	<ul style="list-style-type: none"> <li>Sophisticated equipment not required</li> <li>Low energy for formulation</li> <li>Feasible</li> <li>Less particle aggregation</li> <li>Scale up easily</li> </ul>	<ul style="list-style-type: none"> <li>Suitable for low lipid content</li> <li>High surfactants required</li> </ul>	(230-238)
Membrane contractor technique	In an aqueous surfactant solution, via membrane passing of drug containing lipid melt forms the emulsion. Upon cooling of emulsion, results in the formation of nanoparticles	<ul style="list-style-type: none"> <li>Highly scale up process</li> </ul>	<ul style="list-style-type: none"> <li>Higher variation from batch to batch</li> <li>Frequent replacement of membrane</li> </ul>	(230-238)
Coacervation Phase separation technique	Fatty acids lower pH in presence of the stabilizer causes the precipitation of nanoparticle	<ul style="list-style-type: none"> <li>Feasible</li> <li>Sophisticated equipments not required</li> </ul>	<ul style="list-style-type: none"> <li>Suitable for alkali salts containing lipids</li> <li>pH sensitive drugs are not suitable</li> </ul>	(230-238)
Phase inversion temperature technique	Irreversible cooling shock resulted in the phase inversion	<ul style="list-style-type: none"> <li>avoids use of large amount of surfactant</li> <li>suitable for thermo sensitive drugs</li> </ul>	<ul style="list-style-type: none"> <li>Aggregation of particles</li> <li>Unstable emulsion</li> </ul>	(230-238)

Emulsification solvent evaporation technique	Evaporation	<ul style="list-style-type: none"> <li>• Sophisticated equipments does not required</li> <li>• Suitable for thermo sensitive drugs</li> <li>• Feasible</li> <li>• Lesssize diameter particles can developed</li> </ul>	<ul style="list-style-type: none"> <li>• Toxic effects can be obtained if organic solvent does not removed completely</li> <li>• Agglomeration</li> <li>• Suitable only in case of low lipid containing system</li> </ul>	(230-238)
"Double emulsion" or "multiple emulsion technique"	Solidification of emulsion which leads to the crystalization	<ul style="list-style-type: none"> <li>• Sophisticated equipments does not required</li> <li>• Low energy process</li> </ul>	<ul style="list-style-type: none"> <li>• Suitable only in case of low lipid containing system</li> </ul>	(230-238)
Emulsification-solvent diffusion technique	Diffusion	<ul style="list-style-type: none"> <li>• Sophisticated equipments does not required</li> <li>• Easy to formulate</li> </ul>	<ul style="list-style-type: none"> <li>• Suitable only in case of low lipid containing system</li> </ul>	(230-238)
Solvent injection technique	Formation of the nanoparticles when lipid phase added to aqueous	<ul style="list-style-type: none"> <li>• Sophisticated equipments does not required</li> <li>• Scalability</li> </ul>	<ul style="list-style-type: none"> <li>• Suitable only in case of low lipid containing system</li> </ul>	(230-238)
Supercritical fluid based techniques	Precipitation of nanoparticles	<ul style="list-style-type: none"> <li>• High efficient method</li> <li>• Suitable for thermo and pH drugs sensitive</li> <li>• Organic solvents not required</li> </ul>	<ul style="list-style-type: none"> <li>• Not economical</li> </ul>	(230-238)
Film ultrasonication and dispersion technique	Ultrasonication	<ul style="list-style-type: none"> <li>• Sophisticated equipments does not required</li> </ul>	<ul style="list-style-type: none"> <li>• Film ultrasonication and dispersion technique</li> </ul>	Ultrasonication

## **2.26 Marketed products of the NLCs (238)**

Many of the researchers developed the NLCs in the form of the cream and serum for topical use and those are available in the market, which are listed below

- NLC deep effect eye serum
- Cutanova Nanorepair Q10 Cream
- Extra moist softener
- FloraGlo®
- Nanolipid restore CLR®
- Cutaneous nanovital Q10 cream (238)

## **2.27 DOE**

DOE is a comprehensive and systematic approach to conduct the experiments by using the scientific and statistical principles to aid in the establishment of relationship between input (factors) and output (responses) variables. In another manner it can be defined as cause and effect relationship (239)

DoE is regarded as a critical component for optimization of product and process performance.

### **2.27.1 Advantages of the DoE**

- It gives best formulation
- It is economical because it provides information on product and process performance with few trials.
- Model equations can be used to simulate the behaviour of a product or process. It also aids in the determination of agonistic and antagonistic interactions between the variables
- Modifications to the optimized formulations are simple to incorporate since all response variables are quantitatively guided by a set of input variables

Selection of the DOE is based on the objectives, number of input variables or factors, interactions to be studied, statistical validity and effectiveness of each design. Experimental designs may be divided into 2 types

1. Screening designs which include Plackett-Burman, Fractionate factorial, two level full factorial
2. Optimization designs such as Box-Behnken design , central composite, 3-level factorial design

### **2.28 Box Behnkan design (BBD) design for optimization of formulation**

Among all designs BBD is the most proficient and power full. It comes under response surface methodology. BBD was proposed to achieve the following goals

- Three levels were used to obtain the optimized value for that each independent factor was coded as -1, 0 and +1 (239-240)
- The number of coefficients in quadratic model to the number of experimental points can be achieved.
- The estimated variance can be achieved for designs with 4 and 7 factors. The variance is depending upon the distance from the center.
- For estimation of parameters by using the second order response surface model, it can be generated by combining ideas from incomplete block design or factorial experiments.
- It require 3 levels to run experiment and minimum of three factors.
- BBD design is used to optimize the min effect, interaction effect and quadratic effect (239-240)

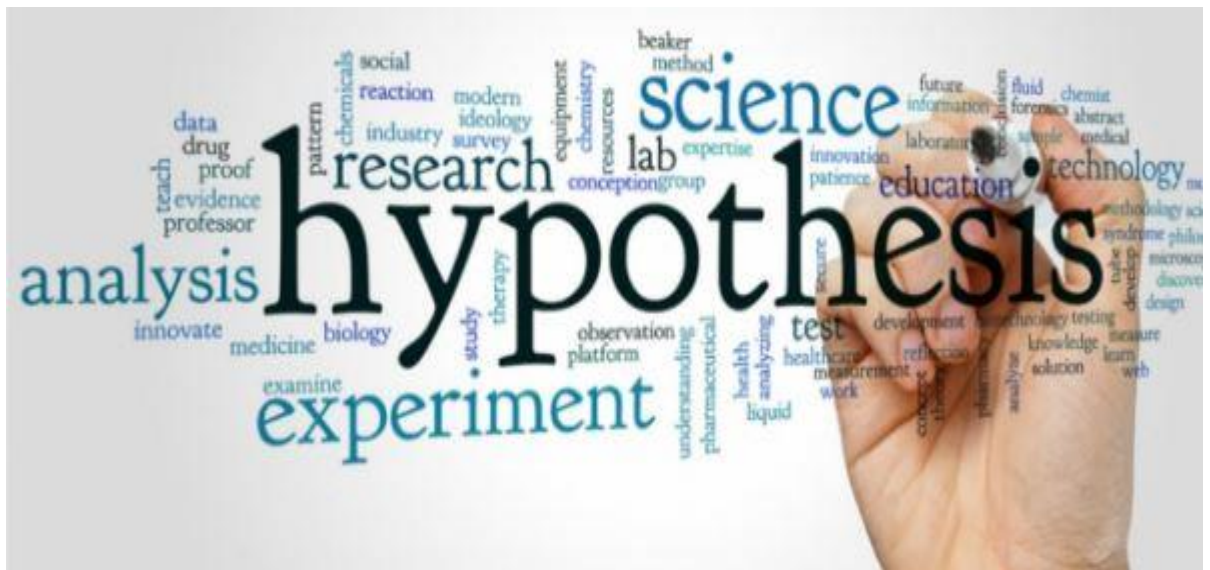
To achieve said goals, in current study, BBD was selected in the optimization of formulation, to ensure the accuracy and quality of the procedure and product, respectively. As a result there have been few studies that have used BBD for optimization of NLCs development are summarized in Table 14



**Table 14.** Few studies that have used BBD for optimization of NLCs

Drug	Input variable	Response	References
Maduramicin ammonium	"Solid lipid (SLs) VS solid-liquid mixed lipid ratios" (%) "Emulsifiers VS solid-liquid mixed lipid ratios" (%) "Drug VS solid-liquid mixed lipids ratios" (%)	Hydrodynamic diameter (nm) Zetapotential (ZP) (mV)	(241)
Ceritinib	Concentration of L- $\alpha$ -Phosphotidylcholine (w/v) Concentration of Chitosan (w/v) Concentration of palmitic acid (w/v)	Poly dispersity index (PDI) Particle size (PS) (nm)	(242)
Apremilast	Amount of lipid Surfactant concentration (SRC) Sonication time (ST)	PS, % entrapment efficiency (%EE)	(243)
<i>Smilax china</i> and <i>Salix alba</i>	Total lipid concentration Total surfactant concentration ST	PS (nm)\ PDI, %EE	(244,245)
Curcumin	SL-LL ratio SRC Ultrasonication time	PS (nm) % EE % Drug release	(245,246)
Triamcinolone acetonide	LL concentration to total lipid Amount of SR Amount of drug	PS (nm) EE (%)	(247)
Methotrexate	Amount of LL (mg) Amount of SR (mg) Amount of drug (mg)	PS (nm) PDI %EE	(248)
Silymarin	SL to LL SRC (% v/v) Homogenization speed (rpm)	PS % EE	(249)
Bicalutamine	Total lipid (%) LL (%) Soyalecithin (%)	PS EE (%)	(250)
Candesartan cilexetil	Lipid to drug ratio SL to LL ratio SRC (%)	PS (nm) ZP (mV) EE (%)	(251)

# Chapter 3



# Hypothesis

### 3. Hypothesis

The current treatments for psoriasis show the utilization of topical and oral drug delivery systems but the patients are not satisfied with that therapy due to symptomatic relieve, remission and severe side effects, frequent reoccurrence and poor efficacy of the therapy causes discontinuation of the treatment. The most accepted treatment from a clinical view is biological however, it is not economical. Thorough literature search related to psoriasis condition aid in the identification of further improvements and requirement of formulation development.

In that scenario, existing literature survey, showing the herbal drugs as well as the probiotics has promising effects toward the management of the psoriasis. Hsin-Ju Li *et al.* in 2020 studied “the efficacy of CS, which is plant flavonoid. It has anti-inflammatory and antioxidant effects for psoriasis. The study of CS on IMQ induced mouse model significantly decreased skin lesions of psoriasis and skin barrier disruption. Moreover, the scientific reports concluded that the “CS reduced “IL-22”-induced CCL20 (CC motif-Ligand 20), “IL-17A”, “TNF- $\alpha$ ”, and antimicrobial peptide release from epidermal keratinocytes in mice”. (252)

David Groeger *et al.* in 2013 studied the effect of “*Bifidobacterium longum*” subsp. *BI* 35624, clinically by oral administration, they concluded that, it has the immune regulatory effect in the gut as well as beyond intestinal sites. Along with it also represented the improved regulatory T cells in healthy human volunteer’s blood samples. Significantly reduced plasma c-RP levels, IL-6 and TNF- $\alpha$ , in psoriatic patient’s groups, as well as in patients with chronic fatigue syndrome and ulcerative colitis. (253)

In 2011 Patrycja Konieczna *et al.* found that *BI* upon oral ingestion to humans specifically promoted immune-regulatory responses by the induction of IL-10 secreting T-cells and FOXP3.(254) It demonstrated that *BI* has therapeutic action in inflammatory disease patients.

The present research work was intent to co-administer the CS and *BI* for anti-psoriatic activity. However, formulation development selection and route of administration is based on the physicochemical nature of the drug and type of disease. Looking at the

challenges associated with CS such as water insolubility nature and pre systemic metabolism upon oral administration. The topical route of administration was selected it could overcomes that challenges associated with CS

A thorough analysis of factors influencing the psoriatic skin, which include dry skin, sensitivity, imbalanced lipids, thickened inflammed skin with excessive differentiation of corneocytes. In addition to above mentioned factors, poor penetration of drugs through stratum corneum, increased levels of cholesterol, decreased levels of ceramides and reduction of natural moisturizing agent also influences the topical delivery of drugs. It is required to select the suitable drug delivery system to manage the psoriatic condition. NLCs offer a unique solution to psoriasis patients by skin hydration and increasing the permeability of drugs via skin (230). NLCs are considered as the versatile, latest generation lipid nanoparticles, due to presence of high drug loading capacity and entrapment efficiency.

In current study for delivery of both herbal drug i.e CS and PB (*BI-UBBI-01*), in the form of nano-formulation systems, NLCs are selected for the topical delivery in the effective treatment of psoriasis. The formulation was hypothesised to develop by the modified hot homogenisation method followed by the ultra and probe sonication. It was given the Figure 7

### **3.1 Aim**

The present study deciphers “Development and evaluation of topical nanoformulation of chrysin and probiotic for psoriasis”

### **3.2 Objectives**

- To prepare optimized *Chrysin* loaded Nanostructured lipid carriers (CS-NLCs)
- To incorporate the optimized *Chrysin* loaded Nanostructured lipid carriers and probiotic in a secondary vehicle
- To characterize the developed formulation
- To evaluate the anti-psoriatic activity of developed formulation using animal model

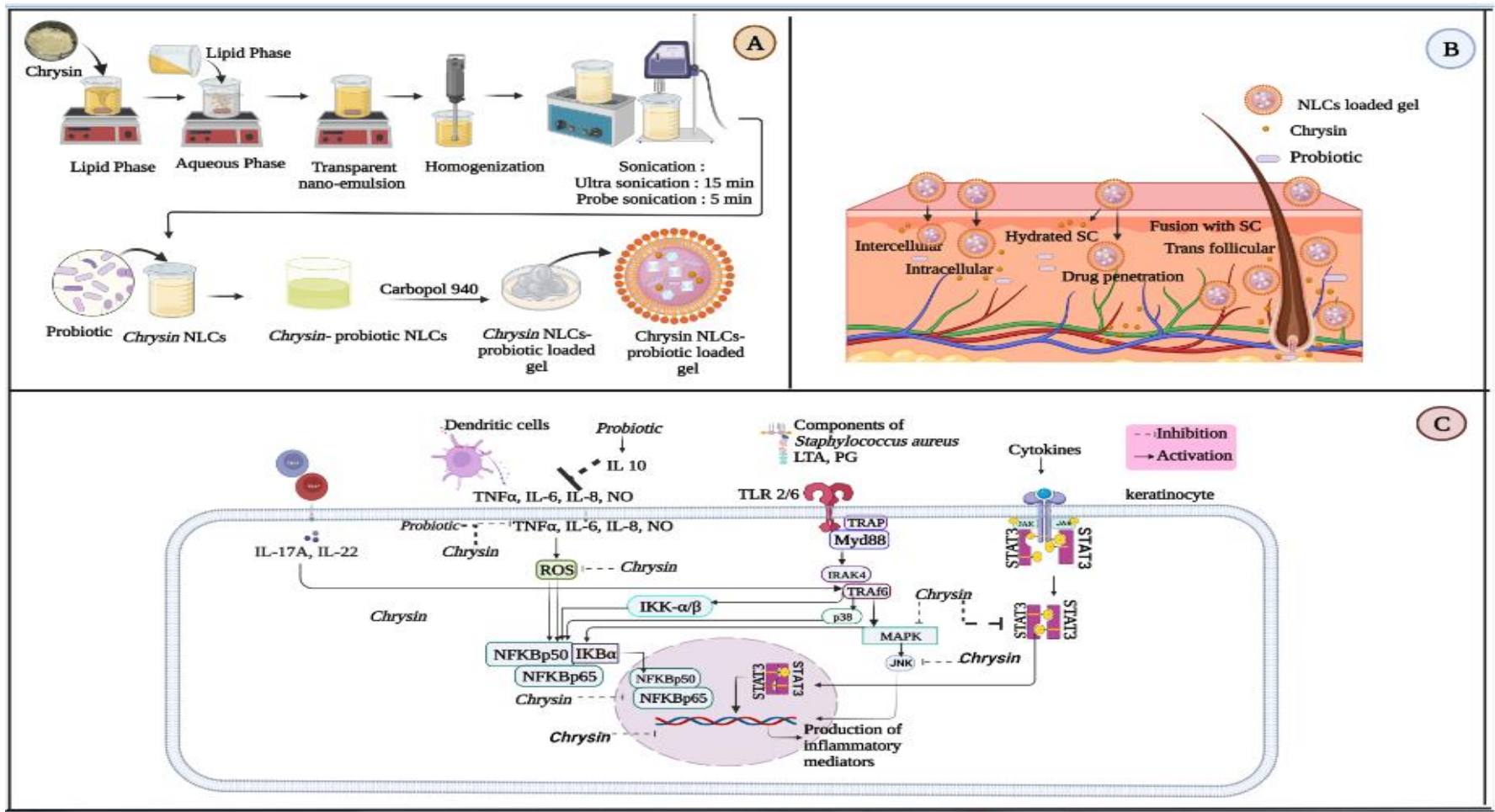


Figure 7. Hypothesis of the research

# Chapter 4



## Experimental work

## 4. Experimental work

### 4.1 Materials

The list of chemicals and instruments employed in study were enlisted in the below mentioned Table 15 and Table 16

**Table 15.** List of chemicals

Chemical name	Source
Acconon MC8-2EP/NF	IMCD Pvt. Ltd, India
Acetonitrile HPLC Grade	Loba Chemie Pvt.Ltd., India
Betamethasone Valerate Skin cream BP	GlaxoSmithKline Pharmaceutical Ltd., India
Buffer capsules pH 6.8 ± 0.05	Merck specialities Pvt. Ltd., India
Buffer capsules pH 7.4 ± 0.05	Merck specialities Pvt. Ltd., India
<i>Bifidobacterium infantis</i> -UBBI-01 (BI)	Unique Biotech, Hyderabad, India
<i>Chrysin</i> (active pharmaceutical ingredient)	BLD Pharma tech Pvt. Ltd India (>98.0 % purity)
Citric acid	Loba Chemie Pvt.Ltd., India
Compritol® 888 pellets	Gattefosse Pvt. Ltd., India
Capryol 90 (C90)	Gattefosse Pvt. Ltd., India
Capmul MCM	IMCD Pvt. Ltd, India
Carbopol 940	Loba Chemie Pvt.Ltd., India
“Chloroform”	Loba Chemie Pvt.Ltd., India
Dialysis membrane 120KD	Himedia Lab Pvt. Ltd., India
Diethyl ether	Loba Chemie Pvt.Ltd., India
Ethanol	Central Drug House Pvt. Ltd., India
Emulcire 61 <sup>TM</sup> WL 2659 pellets (EM-P2659)	Gattefosse Pvt. Ltd., India
Formaldehyde	Meru Chem Pvt. Ltd. India.
Formic acid	Loba Chemie Pvt.Ltd., India
Glyceryl monosterate (GMS)	Mohini Organics Pvt. Ltd., India
Gelucire® 44/14(GS-44/14)	Gattefosse Pvt. Ltd., India
Gelucire® 43/01 pellets (GSP-43/01)	Gattefosse Pvt. Ltd., India
Geleol (GL)	Gattefosse Pvt. Ltd., India
Hexane	Central Drug House Pvt. Ltd., India
Isopropyl myristate (IPM)	Mohini organics Pvt. Ltd., India
Imiquimod cream	Glenmark Pharmaceuticals India
Labrafil M 1944CS	Gattefosse Pvt. Ltd., India
Lauroglycol FCC (L-FCC),	Gattefosse Pvt. Ltd., India

Labrafac labrafil WL1349 (LB-WL1349)	Gattefosse Pvt. Ltd., India
Labrasol	Gattefosse Pvt. Ltd., India
Migolyol 812 (M 812)	Mohini organics Pvt. Ltd., India
Nutrient agar	Himedia Laboratories Pvt. Ltd., India
Octonol	“Central Drug House” Pvt. Ltd., India
Oleic acid (OA)	Mohini organics Pvt. Ltd., India
Palmitic acid	Gattefosse Pvt. Ltd., India
Poloxamer 188	IMCD Pvt. Ltd, India
Poloxamer 407	IMCD Pvt. Ltd, India
Paraffin oil (PO)	Loba Chemie Pvt.Ltd., India
Rhodamine B	Loba Chemie Pvt.Ltd., India
<i>Staphylococcus aureus</i>	IMTECH Chandigarh
Sodium bicarbonate	Loba Chemie Pvt.Ltd., India
Sodium chloride (99.5% extra pure)	Loba Chemie Pvt.Ltd., India
Sodium hydroxide	Loba Chemie Pvt.Ltd., India
Stearic acid (SA)	Gattefosse Pvt.Ltd., India
Soyabean oil (S-O)	Loba Chemie Pvt.Ltd., India
Tween 80 (T80)	Mohini organics Pvt. Ltd., India
Transcutol HP	Gattefosse Pvt. Ltd., India
Tween 20	Loba Chemie Pvt.Ltd., India
Veet hair removal cream	Reckitt Benckiser; India.



**Table 16.** List of instruments

<b>Instruments name</b>	<b>Manufacturer</b>
Centrifuge	Remi Electrotechnical., India
Diode array detector	SPD-M20A Shimadzu Japan
Differential Scanning Calorimeter	Perkin Elmer, USA
Deep freezer	Blue star Ltd., India
Electronic weighing balance	CY360, Shimazu Co.Ltd., Kyoto, Japan
Fourier transform infrared spectrophotometer (FTIR)	Perkin Elmer, USA
High performance liquid chromatography (HPLC)	Shimadzu LC-20AD Prominence.CO. Ltd., Japan
High resolution Transmission electron microscopy (HR-TEM)	JEM-2100 plus electron microscopy, Jeol, Japan
Hot air oven	Cadmach Drying Oven, Cadmach Machinery Ltd., India.
“Homogenizer”	Glass- Teflon potter homogenizer, Thomas scientific, USA
HEPA filters	Sidco filter company, USA
Lyophilizer	BTI-10 FDMN, Biotechnologies Inc., India.
Magnetic stirrer	Remi 5 MLH,India
Malvern Zeta sizer	Nano ZS90, UK
Optical microscopy	KYOWA-Getner Instruments Pvt.Ltd., Japan
Probe Sonicator	Lab man Scientific Pvt. Ltd., India
P <sup>H</sup> meter	Phan, Lab India
Probe sonicator	Labman Scientific Pvt. Ltd., India
0.45 µm syringe filter	Merck Germany
0.22 µm syringe filter	Merck Germany
Scanning electron Microscopy	Jeol JSM-7610F plus, Japan
Shaking water bath	Labfit, India
UV-visible spectrophotometer	UV-1800, Shimazu Co. Ltd., Japan
Ultra bath sonicator	LabFit, India
X-ray diffractometer	Bruker D8, USA

## **4.2 Proposed methodology**

### **4.2.1 Plan of work**

#### **4.2.1.1 Pre formulation studies**

##### **4.2.1.1.1 Physical examination**

All the raw materials required for the research study was collected, then about 1gm of CS was taken on petriplate. The colour, physical nature and odour of the drug was analysed via visual observation. The screening was carried for the presence of the foreign particle in it.

##### **4.2.1.1.2 Determination of wavelength maxima ( $\lambda_{\max}$ ) of CS**

The wavelength maxima of CS was examined by using UV-visible spectrophotometer (UV-1800 Shimadzu-spectrophotometer (Kyoto JAPAN)). For that 10 mg of CS was dissolved in the 1mL of methanol containing in 10 mL capacity volumetric flask and then final volume made up to the mark, to obtain the 1000  $\mu\text{g/mL}$  concentration (stock solution-1). From stock solution-1, 0.1 mL of standard solution was withdrawn into the 10 ml capacity volumetric flask and then further diluted with methanol up to the mark to obtain standard solution of 10  $\mu\text{g/mL}$  concentration. This standard solution in the range of 400-200 nm was scanned against methanol as blank using UV-visible spectrophotometer. (255,256)

##### **4.2.1.1.3 RP-HPLC method development and validation of CS**

###### **4.2.1.1.3.1 Selection of optimized mobile phase ratio for estimation of CS**

For the estimation of CS, different trials by varying the mobile phase ratios (50:50 v/v, 60:40 v/v & 70:30 v/v) of methanol (HPLC grade) and 0.1% Formic acid (FA) in water in isocratic elution was carried out. Followed by analysis of system suitability and retention time ( $R_t$ ) of CS. Based upon the findings, a suitable mobile ratio was optimized for method development

###### **4.2.1.1.3.2 Method development for estimation of CS by RP HPLC**

The HPLC system consist of a LC solution software, photodiode array detector (SPDM20A; Shimadzu, Japan), a 20 $\mu\text{L}$  loop (Rheodyne), and mobile phase delivery pump (LC-20 AD; Shimadzu, Japan). A C-18 (Nucleodur C<sub>18</sub>, 250 mm

×4.6mm i.d.,5 $\mu$ , 100 A<sup>0</sup>) reverse phase column was utilized for separation of CS using methanol and 0.1% FA in water (70:30 v/v) as mobile phase. The rate of flow was 1 mL/min and CS was detected at the wavelength of 268 nm. 2, 4, 6, 8 and 10  $\mu$ g/mL of standard solutions were prepared in methanol and analysed. As per the ICH Q2 (R1) guidelines developed method was validated (257)

#### **4.2.1.1.3.3 Standard solution preparation**

In order to get the 1000  $\mu$ g/mL concentration of the standard stock solution, 100 mg of CS dissolved in 100 mL of methanol. A 10 mL of solution was pipette out from standard stock solution and diluted up to 100mL with methanol to get solution of known concentration of 0.1mg/mL. Further dilutions are carried out from 0.1 mg/mL to get the 2, 4, 6, 8, 10  $\mu$ g/mL standard solutions in to 10 mL volumetric flask with methanol (258)

#### **4.2.1.1.3.4 Method validation**

Accuracy, linearity, robustness, precision, limit of detection (LOD) and limit of quantification (LOQ) (258) are the parameters used for the validation of the developed method

#### **4.2.1.1.3.5 Linearity and range**

The calibration curve of CS was plotted by taking the mean peak area (5 replicates) on y –axis and concentration on x-axis (258)

#### **4.2.1.1.3.6 Accuracy**

Accuracy study of CS was performed on the basis of absolute recovery at three levels 80 %, 100 %, and 120 % of 6  $\mu$ g/ mL sample concentration. These levels are labelled as LQC, MQC, and HQC respectively. To prepare these dilutions, aliquots of 4.8, 6, and 7.2 mL were withdrawn from 10  $\mu$ g/mL of stock solution and transferred individually into 10 mL standard volumetric flask. Finally, volume of each of the flasks was adjusted to 10 mL with mobile phase. Each study was performed five times and mean values were collected. The following equation was used for calculating the percentage absolute recovery.(258-260)

$$\text{Absolute percent recovery} = \frac{\text{Actual concentration recovered}}{\text{Theoretical concentration}} \times 100 \text{-----Equation 1}$$

#### **4.2.1.1.3.7 Precision**

Precision is defined as the degree of agreement between category of samples whenever an analytical method is applied to a number of samplings of uniform sample. Repeatability and intermediate precision (formerly ruggedness) were used to verify developed method was precise or not. Repeatability was evaluated by injecting five times the samples of LQC, MQC, and HQC on the same day' (intraday). In order to carryout the intermediate precision, five injections of LQC, MQC, and HQC were given on three consecutive days (interday) as well as by three different analysts (interanalyst). The mean results were recorded, and %RSD was calculated (258-260)

#### **4.2.1.1.3.8 Robustness**

Robustness is the reliability measure of an analytical developed method that is unaffected by small intended variations in method process variables. The analytical method was studied by varying the flow rate (0.8, 1.0 and 1.2 mL/min), wavelength (266, 268 and 270 nm), different lots of column (34507023, 34507034 and 34505023), ratios of mobile phase (68:32,70:30 and 72:28) and concentration of FA (0.1% v/v, 0.8% v/v and 0.125 v/v) (260,261)

#### **4.2.1.1.3.9 Estimation of LOD and LOQ**

LOD is defined as the lowest analyte concentration in a sample which can be identified but not quantified (260). The lowest amount of an analyte in a sample can be quantified with acceptable precision and accuracy (260) in case of LOQ. LOD and LOQ was determined by using  $3.3\sigma/S$  and  $10\sigma/S$  equations, in which 'S' is the slope of the calibration curve, sigma is the standard deviation of response. Standard deviation of Y intercepts of regression line was used as standard deviation (260,261)

#### **4.2.1.1.3.10 System suitability**

System suitability testing (SST) is most frequently used to certify chromatographic system resolution, column effectiveness, and reproducibility, which conforms its

flexibility for specific analysis. All the equipment, electronics, analytical operations and samples can be analysed or can be evaluated whole, as it is the integral part of the system. The system suitability parameters were evaluated. (260,261)

#### **4.2.1.1.3.11 Specificity**

In order to determine the specificity of CS in presence of adjuvants used for development of NLCs various solvents which include methanol, glycerol monostearate (GMS) Emulsire 61, Tween 20 (T-20) and Transcutol HP (T-HP) were injected in to the HPLC and their interference at the retention time of drug was noted (260,261)

#### **4.2.1.2 Characterization of CS**

##### **4.2.1.2.1 Fourier transform infrared spectroscopy (FTIR) study of CS**

The FTIR (Perkin Elmer) study of CS was performed by the direct sampling method. For that 1-2 mg of CS was scanned in between the 4000-400  $\text{cm}^{-1}$  wave number region. Then the structural conformation of the CS was determined by the different functional groups displayed in the form of bands (262)

##### **4.2.1.2.2 Differential scanning calorimeter study (DSC) of CS**

The “CS” melting point was estimated by the Perkin Elmer DSC apparatus. For that powdered sample of CS (2-3mg) was taken in aluminium pan, heated over the range of 30-440 °C at rate of 10 °C/min using a dry-nitrogen flush at flow rate of 19.8mL/min. The empty aluminium pan was taken as reference (263,264)

##### **4.2.1.2.3 X-ray diffraction (XRD) analysis**

The physical nature of CS was determined by the x-ray diffractometer (Bruker AXS, D8 Advance, Germany). It was scanned with the monochromatized Cu-K $\alpha$  radiation at the speed of 2°C /min from 2-50 °C with the 2 $\theta$  angle at the voltage of 40Kv and 30MA (265-266)

##### **4.2.1.3 Partition co-efficient study of CS**

The partition co-efficient of a CS was determined by taking equal volume of (25ml) of n-octanol and water in separating funnel after the addition of 6.25 mg of CS. The liquid mixture was shaken for 8 h with the time gap of 60 min in alpha mode.

The clear liquids were separated after 24 h, then filter the liquids by 0.25µm membrane filter. Followed by the samples were analysed by UV spectrophotometer (1800 shimadzu, Japan) (267-268)

#### **4.2.1.4 Preliminary screening of SLs, LLs and SR based on solubility of CS**

##### **4.2.1.4.1 SLs**

The solubility of CS in various SLs (Stearic acid, palmitic acid, Compritol 888 pellets, Glyceryl monostearate, Gelucire 44/14, Geleol, Emulcire 61™ 2659 pellets, Gelucire 43/01 pellets) was estimated by adding CS (approximately 50 mg) in increasing amount from 1 mg till, it failed to dissolve in it in molten form. For that the selected SL was taken in a beaker separately and placed on a magnetic stirrer at 100 °C (269-270). The placed lipids were melted at 10°C above their melting point. The lipid containing drug was immediately transferred into 10 mL stoppered glass vials. To achieve equilibrium (50-60 rpm), vials were placed in shaking water bath for 72 h at 37 ± 0.5 °C. After 72 h, the test tubes were removed from shaking water bath and then diluted with 9 mL of methanol or solvents in which drug as well as excipients are soluble. The diluted samples were then injected to HPLC and area was recorded after passing through 0.45 µm membrane filter. The type of SL required for solubilisation of CS was determined based on the highest solubility of CS in the used lipid.

##### **4.2.1.4.2 LLs and SRs**

The solubility of CS was determined by the addition of known excess amount of the CS (approximately 50 mg) into each of the selected LLs (Capryol 90, Soyabean oil, Acconon MC8, Oleic acid, Lauroglycol FCC, Paraffin oil, Migolyol 812, Labrafac Labraphil WL1349, Capmul MCM (1mL) and SRs Tween 20, Tween 80, Labrasol, Transcutol HP, poloxamer 407, Poloxamer 188 (1mL) into 10 mL stoppered glass vials and vortex thoroughly. To achieve equilibrium (50-60 rpm), vials were placed in shaking water bath for 72 h at 37 ± 0.5 °C. After the achievement of equilibrium the supersaturated solutions were subjected to centrifugation at 10000 g for 20 minutes to separate the un-dissolved portion. The obtained supernatants layers were separated, followed by filtered via 0.45µm membrane filter and finally CS was quantified in each excipients filtrate using RP HPLC at 268 nm after appropriate

dilution with methanol (271). Then the amount of CS solubilised in each excipient was calculated.

#### **4.2.1.4.3 Solubility of CS in the phosphate buffers and hydro-alcoholic solutions.**

The solubility study of CS in the phosphate buffer (pH 6.8, 7.4, 5.5) and hydro-alcoholic (10% and 5% hydroalcoholic solution) solutions was determined as procedure mentioned in the section 4.2.1.4.2 by taking the one mL of the each solvent. Followed by amount of CS solubilised in each solvent was calculated after analysed by the RP-HPLC

#### **4.2.1.5 Compatibility studies**

To evaluate the interaction between the CS and selected excipients viz. GMS, C-90, T20, THP and their subsequent physical mixture, the studies were conducted employing FTIR spectrophotometer (Perkin Elmer). For that 1-2 mg of CS and excipients were scanned over a wave number region of 4000-400  $\text{cm}^{-1}$  by direct sampling method (272).

#### **4.2.1.6 Formulation development**

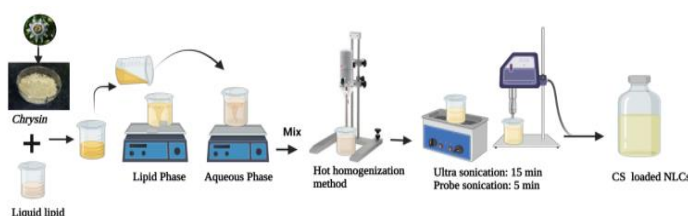
##### **4.2.1.6.1 Initial screening of formulation**

###### **4.2.1.6.1.1 Construction of pseudo ternary phase diagram (PTPD)**

To know the existence of the nanoemulsion region PTPD was constructed using aqueous titration method. As per solubility study results GMS was selected as SL, C90 as LL, T20 as SR and T-HP as co surfactant. The proportion of components required for formation of nanoemulsion region is depends upon quantity of water required for emulsification. For that ratio of components was determined by the aqueous titration method. Initially, the mixture of lipids considered as L<sub>mix</sub> and surfactants considered as S<sub>mix</sub> were taken in the ratios of 1:1, 2:1 and 3:1 (External ratios). Further, within the selected two lipids (L<sub>mix</sub>) and two surfactants (S<sub>mix</sub>), various ratios (internal ratios) were taken, which resulted in nine prototype formulations in each external ratio (273)

#### 4.2.1.6.2 Modified hot homogenization technique followed by ultra and probe sonication method

The NLCs were prepared by using modified hot homogenisation method, followed by ultra and probe sonication. For that lipid soluble ingredients and water soluble ingredients were taken separately in a beaker, briefly the selected solid lipid (GMS) was heated above the melting point (80°C) of it on magnetic stirrer at 2000 rpm. To that drug (CS:0.005%) solubilised liquid lipid (C-90) mixture was added. Then this lipid mixture was added to the aqueous mixture containing surfactant (T-20), co surfactant (T-HP) and water. This homogeneous mixture was further added to the 200 mL hot water drop wisely under the homogenization (4000 rpm) up to 45 min, followed by ultra (15 min) and probe sonication (5 min) to improve its micromeritics (274). Briefly ultrasonication reduced the particle size from micron to submicron level, whereas probe sonication reduced the particle size from submicron to nano size range. Then developed formulation was kept aside for the formation of NLCs. The diagrammatic representation of CS loaded NLCs are shown in the Figure 8



**Figure 8.** Diagrammatic representation of the CS loaded NLCs development by modified hot homogenization followed by ultra and probe Sonication

From the results of ternary plot diagram, 6 transparent nano emulsions were selected for further conversion into CS loaded NLCs. Further, they were analyzed for PS, PDI, ZP, % EE and percentage drug loading (% DL).

#### 4.2.1.6.3 DoE

##### 4.2.1.6.3.1 BBD design

From the results of the developed CS loaded NLCs, which was obtained from the selected nanoemulsion regions of ternary plot diagram in various ratios it was understood that the CS loaded NLCs in that range have better PS, ZP, %EE and %DL.



Hence, to have better insight about the impact of all components used in formulation of CS loaded NLCs on PS, ZP, % EE a randomized 4 factor 3 level BBD (DoE, Stat/Ease USA version 11.1.2.0.lic) was used. For that four independent variables i.e amount of GMS (SL), C-90 (LL), T-20 (SR) and T-HP (Co-surfactant) were taken. Each independent factor was screened at three levels, which were designated as -1, 0 and +1. Three responses were recorded for the study which includes PS, ZP and % EE. A total 29 runs (formulations) were designed by the software. The quantity of CS (0.005%) kept constant in all 29 formulations (274). All the test experiments were carried in random manner to improve the predictability of the model. The design level and independent factor are shown in Table 17

**Table 17.** The design level and independent factors

Factor	Levels		
	-1	0	+1
A: Solid lipid (mg)	50	125	200
B: Liquid lipid (mg)	25	62.5	100
C: Surfactant (mg)	400	550	700
D: Co-surfactants (mg)	225	337.5	445

#### 4.2.1.6.3.2 Development of CS loaded NLCs

To prepare CS loaded NLCs by using modified hot homogenization technique followed by ultra and probe sonication which was mentioned in detailed in section 4.2.1.6.2 The weights of GMS, C-90, T-20 and T-HP were taken as per the quantities given by DoE for each batch (200 mL) which was shown in Table 18

**Table18.** Quantities as per DOE

Formulations	Independent variables			
	GMS (mg)	C-90 (mg)	T-20 (mg)	T-HP (mg)
F1	125.0	62.5	400.0	450.0
F2	125.0	62.5	550.0	337.5
F3	200.0	62.5	550.0	225.0
F4	50.0	62.5	550.0	450.0
F5	200.0	62.5	700.0	337.5
F6	125.0	62.5	400.0	225.0
F7	200.0	62.5	400.0	337.5
F8	125.0	25.0	550.0	225.0
F9	200.0	62.5	550.0	450.0
F10	50.0	100.0	550.0	337.5
F11	50.0	25.0	550.0	337.5
F12	50.0	62.5	400.0	337.5
F13	125.0	62.5	550.0	337.5
F14	125.0	100.0	700.0	337.5
F15	125.0	62.5	550.0	337.5
F16	50.0	62.5	700.0	337.5
F17	125.0	100.0	550.0	450..0
F18	50.0	62.5	550.0	225.0
F19	200.0	25.0	550.0	337.5
F20	125.0	62.5	700.0	450.0
F21	125.0	62.5	700.0	225.0
F22	125.0	25.0	400.0	337.5
F23	125.0	100.0	400.0	337.5
F24	125.0	62.5	550.0	337.5
F25	125.0	100.0	550.0	225.0
F26	125.0	25.0	700.0	337.5
F27	125.0	62.5	550.0	550.0
F28	125.0	25.0	550.0	550.0
F29	200.0	100.0	550.0	550.0

#### **4.2.1.6.3.3 Preparation of optimized CS loaded NLCs**

As per the BBD, the obtained SL, LL, SR and co surfactant optimized values were found to be 199.99, 33.92, 700 and 376.869 mg respectively. By using the above-mentioned quantities an optimized CS loaded NLCs were developed by using the procedure as mentioned in the section **4.2.1.6.2**

#### **4.2.1.7 Characterization of developed CS loaded NLCs**

##### **4.2.1.7.1 Morphological study of CS loaded NLCs**

###### **4.2.1.7.1.1 Scanning electron microscopic (SEM) study of CS loaded NLCs (CS-NLCs)**

The morphological study of CS NLCs were determined by using the SEM. For that the formulation was diluted in the ratio of 1:500 (v/v) in double distilled water. A drop of the formulation was placed on silica wafer, which was mounted on a metallic support with use of carbon tape. Then metallic support was kept under desiccator overnight for drying. These sample was scanned after coated with gold (10 nm) under an argon atmosphere (276-277)

###### **4.2.1.7.2 PS, ZP and PDI**

The PS, ZP and PDI of the developed CS-NLCs were evaluated by zeta sizer (Nano ZS90, Malvern Instruments Ltd., India). The sample analysis was carried out by passing a dynamic light scattering via sample filled disposable cuvette at 25 °C temperature. The sample was diluted up to 10 times with double distilled water and filtered via 0.2 µm membrane filter before performing the analysis (278). In similar manner, ZP also analyzed by placing same sample in electrode containing cuvette (272-278) each sample was recorded in triplicate with standard deviation.

###### **4.2.1.7.3 Determination of %EE and %DL**

The CS NLC sample was tested for % DL and EE by centrifugation method. Firstly, the initial amount of CS was considered (Angellotti et al., 2021). Consequently, an aliquot of 2 mL of NLCs dispersion was taken in eppendorfs and centrifuged (Remi Elektrotechnik Ltd-India) at 10000 rpm for 15 min to separate the unloaded CS.

The supernatant was collected and diluted with methanol, followed by filtered through membrane filter (0.2 µm cut off) and analyzed by HPLC method mentioned section in 4.2.1.1.3.2 at 268 nm. The % EE of CS was calculated by equation 2 and 3 respectively (279)

$$\% \text{ EE} = \frac{\text{Amount of initial drug} - \text{Amount of free drug}}{\text{Amount of initial drug}} \times 100 \text{-----Equation (2)}$$

The amount of CS loaded in the lipids was determined by using Equation (3)

$$\% \text{ DL} = \frac{\text{Weight of initial drug} - \text{weight of free drug}}{\text{weight of total lipid}} \times 100 \text{-----Equation (3)}$$

#### **4.2.1.8 Development of CS-PB NLCs**

For the development of CS-PB-NLCs, to the optimized (mentioned in section no 4.2.1.6.3.3) CS NLCs 0.2 g of probiotic i.e. BI (contains 10 billions CFU of BI) was added and mixed to prepare CS-PB NLCs.

##### **4.2.1.8.1 Characterization of CS-PB NLCs**

###### **4.2.1.8.1.1 PS, PDI and ZP**

The PS, ZP and PDI of the developed CS-PB NLCs were evaluated by zeta sizer (Nano ZS90, Malvern Instruments Ltd., India). The sample analysis was carried out similarly as mentioned in the section **4.2.1.7.2**

###### **4.2.1.8.1.2 Determination of percent drug % EE and DL**

The % DL and % EE of CS was determined in CS-PB NLCs by centrifugation method, the similar procedure and equations were followed as mentioned in the section **4.2.1.7.3**

#### **4.3 Incorporation of the CS-PB NLCs in a secondary vehicle**

For the incorporation of the CS-PB NLCs in secondary vehicle, carbopol 940 (0.5 % w/v), was chosen as the gelling agent after numerous trails with different gelling agents (HPMC, carbopol 934 and sodium CMC). It was chosen based on the visual appearance and consistency property. To prepare a gel 1 g of the carbopol 940 was dissolved in 50 mL of H<sub>2</sub>O, kept overnight for the soaking of the carbopol

Followed by wet gel was added to the 200 mL of the CS-PB NLCs while being stirred on a magnetic agitator at 1500 rpm in order to achieve lump free dispersion gel. Then the pH of the homogeneous dispersion was adjusted to 6.8 by addition of triethanolamine. Finally nanoformulation containing gel was formed (280)

### **4.3.1 Characterization of CS NLCs-PB loaded gel**

#### **4.3.1.1 Appearance**

The developed formulation CS NLCs PB loaded gel was visually observed to find out the colour, physical nature and odour of it.

#### **4.3.1.2 Spreadability**

To determine the gel spreadability glass slide method was used. For that a measured quantity (0.5 g) of gel was placed on to the previously labelled with 1 cm diameter circle on to the glass slide. On that slide another slide was covered and placed the 50 g weight on that slide up to 120 s. After that spreading coefficient/spreading of gel was determined (281) The spreadability of gel was calculated by using  $S = M.L/T$ , Where M= Weight placed on upper slide, L= Length of glass slide, T= Time taken to separate the slides (281)

#### **4.3.1.3 pH**

The pH of the developed formulation was determined by digital pH meter at room temperature in triplicate manner.

#### **4.3.1.4 Viscosity**

The viscosity of CS NLCs PB loaded gel was determined in triplicate by Brook field viscometer. For that a measured (30 g) quantity of the gel was taken into the beaker and placed below viscometer, with spindle no. 94 at different rpm i.e 5,10, 20, 50, 100,120, and 150 was measured in triplicate manner at room temperature (282)

#### **4.3.1.5 Drug content**

A measured quantity of gel approximately 1 g was taken in the test tube and dissolved in 10 mL of the methanol. Followed by it was filtered through the 0.45  $\mu$ m membrane filter. The sample was analysed by RP-HPLC after appropriate dilutions (283)

#### **4.3.1.6 PS, PDI and ZP**

The PS, ZP and PDI of the developed CS NLCs-PB loaded gel was evaluated by zeta sizer (Nano ZS90, Malvern Instruments Ltd., India). The sample analysis was carried out similarly as mentioned in the section **4.2.1.7.2**

#### **4.3.1.7 SEM study of CS NLCs-PB loaded gel**

The morphological study of CS NLCs-PB loaded gel was determined by using the SEM. For that the formulation was diluted in the ratio of 1:500 (v/v) in doubled distilled water. The sample analysis carried out similarly as mentioned in the section **4.2.1.7.1.1**

#### **4.3.1.8 Evaluation**

##### **4.3.1.8.1 *In vitro* diffusion study**

The diffusion profiles of naive CS gel, CS-NLCs and CS NLCs PB loaded gel, were carried out by franz diffusion cell (FDC) with the help of dialysis membrane (Molecular weight 12 KD). For activation of the dialysis membrane, soaked it in 100 mL of pH6.8(Phosphate buffer) for 24 h prior it's usage. The receiver compartment of FDC was filled with 17 mL of phosphate buffer 6.8 and methanol in 7:3 v/v ratio. The previously soaked membrane was kept in between donor and receptor compartment. The donor compartment was filled with 1 g of CS NLCs PB loaded gel, and the total assembly was kept on magnetic stirrer at 150 rpm. The temperature was maintained at 37 °C using thermostatically controlled magnetic stirrer. At fixed intervals of time (0.5, 1, 2, 4, 6, 12, 24, 32, 36, 40, 44 and 48 h) samples were withdrawn (1mL) and sink condition was maintained. The samples were analysed by HPLC to determine the amount of drug diffused. Similar experiments were carried out with naive CS gel and CS NLCs. All the results are recorded in triplicate manner (333)

##### **4.3.1.8.1.1 Release kinetics study of CS-NLCs and CS-NLCs-PB loaded gel**

The release pattern of CS NLCs and CS NLCs-PB loaded gel was evaluated by applying various mathematical models viz “Zero order”, “First order”, “Higuchi”, “Hixson–Crowel” “Weibull plot” and “Krosmeier- Peppas” “equations” (285)

#### **4.3.1.8.2 Antibacterial study of CS NLCs PB loaded gel**

##### **4.3.1.8.2.1 Collection and enumeration of *Staphylococcus aureus***

*Staphylococcus aureus* (7405) pure stain was procured from the Imtech biotech Chandigarh. Then it was inoculated in nutrient broth (sterilized in autoclave 15 lbs temperature 121°C, for 20 min) under aseptic conditions and incubated for bacterial growth at 28-30 °C up to 24-48 h in incubator. The overnight pure *Staphylococcus aureus* (1mL) culture solution was diluted into 9 mL of the saline solution and further diluted by serial dilution. Furthermore, *Staphylococcus aureus* was inoculated on solidified nutrient agar plates by pour plate technique (sterilized in autoclave at 15 lbs temperature 121°C, for 20min) under aseptic conditions. Then the plates were incubated in the incubator up to 24-48 h. The Colonies of *Staphylococcus aureus* was observed visually and photographic images were taken. The formed *Staphylococcus aureus* colonies were counted with help of colony counter and further conformed by the gram staining technique. The whole process was strictly carried out by following the biosafety guidelines 2.

##### **4.3.1.8.2.2 Determination of zone of inhibition (ZOI) of developed formulation**

The ZOI of the optimized formulation was determined by antibacterial studies at lower and higher doses. The study design protocol for zone of inhibition was followed as mentioned in Table 19. It was designed with 8 treatment groups along with normal, experimental and positive control. The study was done in triplicate using 3 petriplates for each group. Each petriplate was containing 15 mL of sterilized agar medium. The plates were kept in an aseptic laminar air flow environment to maintain sterile condition. These petri plates were kept aside for 30-45 min for solidification. After that, 3 bores were made in each petri plate followed by the addition of inoculums of *Staphylococcus aureus* except normal control. The specified dosing as mentioned in the Table 19 was filled into the bores of petriplates and plates were incubated in an incubator for 48 h at 37 °C (286)

**Table 19.** Study design protocol of zone of inhibition

Group no.	Groups	Treatment	Dosage	No. of petriplates
I	Normal control	(not applicable )	solidified agar media	3
<i>Staphylococcus aureus</i> spread on the solidified agar petriplates				
II	Experimental control	<i>Staphylococcus aureus</i> (Test organism)	50 Billions CFU to be spread on the solidified agar media	3
III	Positive control	Marketed Betamethasone cream	0.1% w/w filled in the 3 bores in each petriplate	3
IV	Treatment I	Blank NLCs gel	Filled in the 3 bores in each petriplate	3
V	Treatment II	CS gel	0.006% w/w of CS suspension filled in the 3 bores in each petriplate	3
VI	Treatment III	CS NLCs gel	0.006% w/w of CS NLCs filled in the 3 bores in each petriplate	3
VII	Treatment IV	PB gel	10 Billions CFU BI filled in the 3 bores in each petriplate	3
VIII	Treatment V	CS L-PB gel	0.003% w/w of CS + 10 billions CFU BI loaded filled in the 3 bores in each petriplate	3
IX	Treatment VI	CS H - PB gel	0.006% w/w of CS + 10 billions CFU BI loaded filled in the 3 bores in each petriplate	3
X	Treatment VII	Optimized CS L NLCs - PB gel	0.0025% w/w of CS + 10 billions CFU BI loaded NLCs filled in the 3 bores in each petriplate	3
XI	Treatment VIII	Optimized CS H NLCs - PB gel	0.005% w/w of CS + 10 billions CFU BI loaded filled in the 3 bores in each petriplate	3

Note: L= Low dose; H= High dose; number of replicates (n)=3

#### 4.3.1.8.3 *Ex vivo* skin permeability study

To determine the amount of the drug permeated from CS gel, CS NLCs gel, CS NLCs PB loaded gel. Similar to that of *in vitro* diffusion studies (FDC) *ex vivo* permeability studies were conducted. However in place of dialysis membrane rat abdominal skin was used due to its structural functional similarity with that of human skin (287).

To carry out the *ex vivo* permeability study, healthy Wistar rats abdominal skin was obtained after sacrificing them. Then the skin was treated with isopropyl alcohol and further cleansed with distilled H<sub>2</sub>O. After that the skin was stored in phosphate buffer 6.8. Then the abdominal skin was positioned on the FDC after filled with methanol phosphate



buffer of pH 6.8 (3:7) as diffusion medium in receptor compartment. On the dorsal side of the rat skin each test formulations (CS gel, CS NLCs, Optimized CS NLCs PB loaded gel) containing the equivalent quantity of medication (0.005% w/v) will be applied evenly. The assembly will be positioned on magnetic stirrer at a temperature of  $37 \pm 2^\circ \text{C}$ , the receptor phase will be agitated at 100 rpm. At predefined time intervals (0.5,1,2,4,6,8,10,12,15,18 and 24 h), 1 mL of sample was withdrawn from the receptor chamber and immediately replaced with new medium to maintain the sink condition. All obtained samples were analyzed by HPLC (288). The cumulative amount of drug permeated and flux ( $\mu\text{g}/\text{cm}^2/\text{h}$ ) were calculated using equations 4 and 5 (289)

Cumulative amount of drug permeated =

$$\frac{\text{Concentration } \left(\frac{\mu\text{g}}{\text{mL}}\right) \times \text{dilution factor}}{\text{Area of permeation cell}} \times \text{volume of acceptor chamber} \text{---Equation}$$

(4)

$$\text{Flux} = \frac{\text{Cumulative amount of drug permeated}}{\text{time (hrs)}} \text{-----Equation (5)}$$

#### 4.3.1.8.3.1 Skin deposition study

After performing the *ex vivo* studies, skin deposition studies were conducted (338). The skin from the FDC was removed and then cleansed with buffer to remove excess amount of the drug from the membrane. Later the exposed skin was cut into small pieces and immersed into the methanol for 24 h. Followed by sample was centrifuged for 15 min at 10000 g. The supernatant was separated, filtered through the 0.45  $\mu\text{m}$  size, membrane filter and analysed in HPLC to know the amount of the drug deposited on the skin (290)

#### 4.3.1.8.3.2 Modified draize test

To check the irritable effect of blank gel draize test was performed on the mouse skin. For that 0.5 gm of blank gel was applied on to the shaved area ( $2 \text{ cm}^2$ ) of the mouse up to 14 days (6 animals). After 14 days of application, the presence or absence of the erythema, dryness and oedema in the exposed area of mouse was observed and the scoring was given as per the below mentioned Table 20 (291)

**Table 20.** Scoring scale for draize test

Erythema, dryness and oedema	Reaction
0	No reaction
1	Slightly erythema, dryness and oedema
2	Moderate erythema, dryness and oedema
3	Moderate- severe erythema, dryness and oedema
4	Sever erythema, dryness and oedema

#### **4.3.1.8.3.3 Cellular uptake study /Mechanism of drug penetration (CLSM)**

“ CLSM study” (292) was conducted for performing the cellular uptake of drug in the rat skin. For that Rhodamine B was selected as suitable dye, due its similar log p value as well as lipid nature with that of CS. It was performed, for Rhodamine B suspension as blank and Rhodamine B loaded NLCs, which mimic the similar type of characteristics with that of developed formulation. CLSM study was carried using FDC. The similar procedure was followed as mentioned in *ex vivo* study. However, the study performed up to 24 h (292)

#### **4.3.1.9 In vivo study**

##### **4.3.1.9.1 Anti psoriatic study using an ImQ triggered psoriatic mouse model**

The optimized CS NLCs PB loaded gel, anti-psoriatic activity was evaluated by a suitable ImQ induced mouse model has been selected. To perform animal study, Institutional Animal Ethical Committee approval was taken and approval no. is IAEC/LPU/2021/85. The experimental procedure was conducted, by preferring Swiss Albino mice (8–11 weeks, 20-30 g weighed) of either sex. Animals were purchased from “Lala Lajpat Rai University of Veterinary and Animal Sciences, Hisar”, India. All animals were acclimatized in pathogen-free plastic cages containing rice husk and provided with pellet feed, water, shelter under environmental conditions ( $25 \pm 2^\circ\text{C}$  with 45% relative humidity and 12-hour light and dark cycle) were maintained (288-293).

#### 4.3.1.9.2 Induction of psoriatic lesion

The animals were divided into 11 groups, each group was containing six mice. The body weight of all the animals was noted. The hairs on the dorsal surface of the mice skin (333) was removed from almost 6.25 cm<sup>2</sup> area using hair removing cream. Then the lesions were developed by administering topical dose of 62.5 mg of ImQ cream on skin and 3.5 mg on ear (5% w/w). This cream was applied to the shaved area of all groups except group 1 for seven consecutive days (293). During the induction period all mice were carefully observed for measuring the degree of irritation, erythematic and psoriatic scaling (333)

#### 4.3.1.9.3 Treatment

After induction (7 days), treatment was started and continued for 7 days. The detail of dosing that was applied on experimental animals and study design is mentioned in Table 21 and Figure 9. All animals were carefully monitored at the treatment period to record the degree of reduction of irritation, erythema and psoriatic scaling.

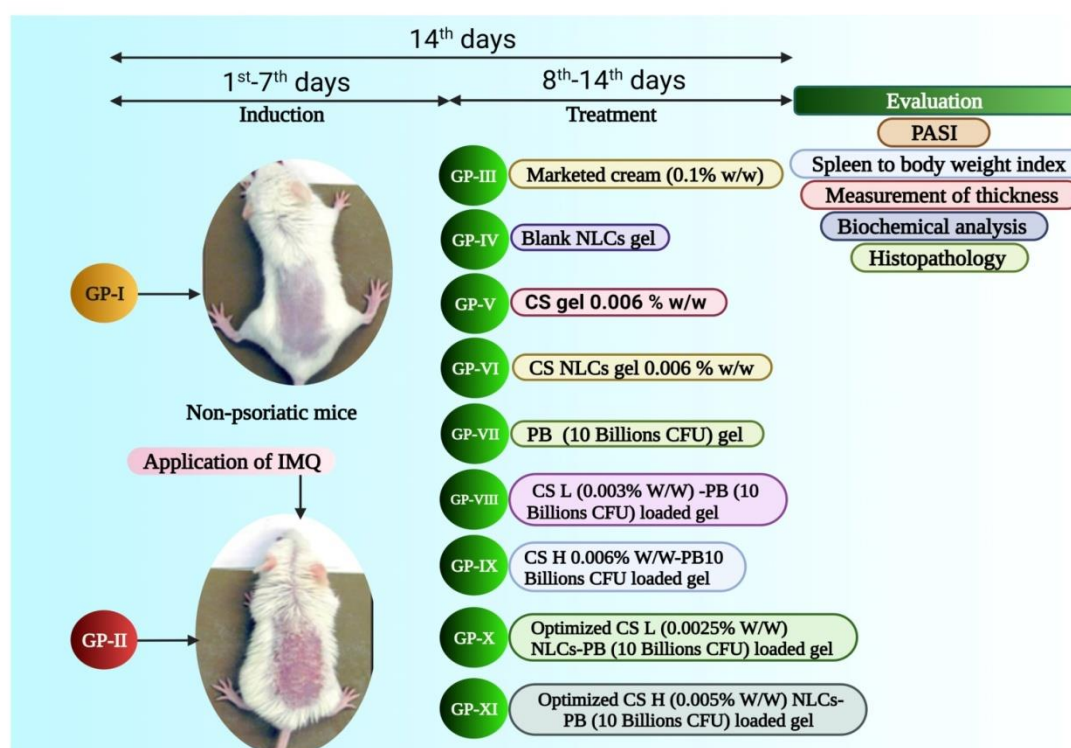


Figure 9. Diagrammatic representation of *in vivo* study design

**Table 21.** Experimental study design

Group no.	Groups (n=6)	Treatment	Dosage and route of administration
I	Normal control	Normal saline (no induction)	To be applied topically once a day for seven days on shaved dorsal surface area for five min
Imiquimod induced psoriasis (5% w/w imiquimod cream daily for 7 consecutive days) (62.5 mg of cream equivalent to 3.125 mg of Imiquimod)			
II	Experimental control	Imiquimod induced cream	5% w/w, dorsal surface of the body one time in a day for “7” days
III	Positive control	Marketed Betamethasone cream on psoriatic skin (Betnovate)	0.1% w/w one time in a day for 7 days after induction of psoriasis
IV	Treatment I	Blank NLCs gel	Applied one time in a day for seven days after induction of psoriasis
V	Treatment II	CS gel	0.006% w/w of CS gel applied one time a day for seven days after induction of psoriasis
VI	Treatment III	CS NLCs gel	0.006% w/w of CS NLCs gel applied one time a day for 7 days after induction of psoriasis
VII	Treatment IV	PB gel	10 Billions CFU BI gel applied one time a day for seven days after induction of psoriasis
VIII	Treatment V	CS L -PB gel	0.003% w/w of CS + 10 billions CFU BI loaded gel applied one time a day for 7 days after induction of psoriasis
IX	Treatment VI	CS H -PB gel	0.006% w/w of CS + 10 billions CFU BI loaded gel applied one time a day for seven days after induction of psoriasis
X	Treatment VII	Optimized CS L NLC –PB loaded gel	0.0025% w/w of CS NLCs + 10 billions CFU BI loaded gel applied once a day for seven days after induction of psoriasis
XI	Treatment VIII	Optimized CS H NLC –PB loaded gel	0.005% w/w of CS NLCs + 10 billions CFU BI loaded gel applied once a day for seven days after induction of psoriasis

L = Low dose, H = High dose

#### 4.3.1.9.4 Evaluation parameters for Antipsoriatic activity

##### 4.3.1.9.4.1 PASI

The severity of inflammation on the dorsal skin was evaluated using PASI scoring tool. The scoring parameters include erythema, scaling, and thickness.

The scoring was given as per the global assessment severity scale which mentioned as follows:

“0”. None (clear) “1”: “Slight” “2”: “Moderate”, “3”: “Marked” and “4”: “Severe”.

(284-333)

The PASI at induction time, then on second, fourth, sixth day of treatment was recorded and at the end of treatment. The PASI reduction, in all groups after treatment and at induction period was noted and compared with each group. Images of all groups dorsal skin was taken before euthanasia of the animal (284-333)

#### **4.3.1.9.5 Ear thickness**

The inflammation in the mouse ear was measured in the form of thickness. For that vernier callipers was used by marking the left ear as control and disease induced was right ear (284-294). Ears images of the all the groups were taken at the time of induction and also during treatment. It has to be taken before euthanasia of the animal (284-333)

#### **4.3.1.9.6 Spleen to bodyweight index**

The body weight of the animals belonging to all groups was noted before induction of psoriasis and again weight has been recorded at period of induction of psoriasis. Afterwards the treatment was started and upon completion of treatment period, the bodyweight of all animals was noted. Followed by with aid of cervical dislocation animals were sacrificed. Then the spleen was collected from each group of mouse, cleaned thoroughly with phosphate-buffer saline and the weight was recorded. Images of collected spleen from all group was captured. The spleen to bodyweight index was also measured (293-297)

#### **4.3.1.9.7 Histopathological studies**

Upon completion of treatment duration, the animals were sacrificed. Then the dorsal skin of and ear was removed from all groups and cleansed with normal saline. The removed specimens were fixed with formalin and then examined for the pathological variations in it. For that specimens were mounted on the slides after staining with heamatoxylin and eosin. The microscopic examination of all prepared group slides was carried out at (10 x and 40 x) magnification. The evaluation parameters such as acanthosis, rete ridges, vascularity, mononuclear cell infiltration, microabscess, pustular formation were performed with help of pathologist. (284-333)

The scoring was as per the following scale (284-333)

Scale	Features
0	No inflammation
1	Mild acanthosis followed by lower inflammatory cells
2	The epidermis thickness was mild and moderate with less inflammatory cells
3	Acanthosis was severd, with rete ridges and higher number of inflammatory cells
4	Very sever acanthosis with pustular formation and micro abscess formation

#### **4.3.1.9.8 Estimation of antioxidant property of developed formulation in skin**

After completion of IMQ induced psoriasis model study, on 15<sup>th</sup> day dorsal skin of mice was collected. A small proportion of skin tissue (phosphate buffer 6.8) from all groups was taken washed and homogenized (tissue homogenizer Type RQ-127A, REMI motors, Vasai, India) separately. Upon completion of homogenization, samples were centrifuged for 10 min in a cooling centrifuge at 10,000 g. After the centrifugation, supernatant was collected and estimated for SOD and catalase levels. In addition to that GSH level was also determined (293-297)

##### **4.3.1.9.8.1 Measurement of catalase activity**

To 20 µL of the supernatant of tissue homogenate, 10 mM of 1 mL of hydrogen peroxide was added in to the cuvette. The reduction of the optical density of the sample was determined by microplate UV spectrophotometer at 240 nm and final amount was determined using calibration curve (293-297)

##### **4.3.1.9.8.2 Levels of SOD**

It consists of preparation of 2 parts. In one part a mixture was prepared by taking the 20 µL (500 mM) of sodium carbonate solution, 2 mL of 0.3 % Triton X-100, 20 µL of (1 .0 mM) ethylenediamine tetracetic acid, 5 mL of hydroxylamine (10 mM), in 178 mL of distilled water. To part 1 mixture 20 µL of supernatant of tissue homogenate was added. To the first part, the second part i.e., 20 µL of C<sub>40</sub>H<sub>30</sub>Cl<sub>2</sub>N<sub>10</sub>O<sub>6</sub> (nitro blue tetrazolium) (240 µm) was added. To the final mixture optical density was

measured at 560 nm for 3 min with time gap of 1 min. The rate of increasing of the optical density was considered as the SOD activity (297)

#### **4.3.1.9.8.3 Estimation of GSH level**

To estimate the levels of the GSH in tissue homogenate, to 20 $\mu$ L of tissue supernatant 180  $\mu$ L of (1 mM) of DTNB (5,5'-dithio-bis (2-nitrobenzoic acid) was added which resulted in yellow colouration. The optical density of the reaction mixture was measured at 412 nm. The concentration was determined from the calibration curve and expressed as  $\mu$ g/mg of protein. (293-297)

#### **4.3.1.9.8.4 Estimation of TBARS**

The skin homogenate was taken to determine the level of lipid peroxidation. For that to a 1 mL of tissue homogenate, 3 mL of 1% H<sub>3</sub>PO<sub>4</sub> (phosphoric acid) solution and 1mL of an aqueous solution of 0.6 % C<sub>4</sub>H<sub>4</sub>N<sub>2</sub>O<sub>2</sub>S (Thiobarbituric acid) was added. Followed by reaction mixture was heated at 80 °C for 45 min, after that cooled in an ice bath. After wards the sample was centrifuged for 10 min at 10,000 rpm. After, that 4.0 mL of n-butanol was added to the separated supernatant to observe the pink colour formation. Followed by the pink colour layer was measured at 532 nm, which is an indication of extent of lipid per oxidation. The standard curve was used for the determination of the exact final concentration (294-297)

#### **4.3.1.9.9 ELISA assay**

##### **4.3.1.9.9.1 Estimation of cytokine levels in the dorsal skin**

After sacrificing the animals, the level of proinflammatory cytokines in the dorsal skin was estimated, which include TNF- $\alpha$  and IL-10. For that the collected skin was cleaned with 0.9 % normal saline and pH 6.8 (phosphate buffer). Then the skin was stored in the 10 % V/V formalin solution at room temperature. The levels of different proinflammatory cytokine were estimated using the protocol directed in ELISA kit (293-297)

#### **4.4 Statistical analysis**

The results were represented in the form of Mean  $\pm$  SEM. The analysis was performed statistically by TWO-way ANOVA followed by multiple comparisons (Post-test- Bonferroni post hoc test) using Graph Pad Prism, version 5 software (294-298). All <sup>a</sup>p < 0.001 as compared with the Experimental control and <sup>b</sup>p < 0.001 as compared with Normal control.

#### **4.5 Stability studies**

According to ICH Q1(R2) the optimized CS loaded NLCs-PB gel was applied to stability studies and conducted at two storage conditions i.e 25  $\pm$  0.2 °C with 65 %  $\pm$  5% RH and 5  $\pm$  3 °C for 6 months. The samples were withdrawn at the end of 3<sup>rd</sup> and 6<sup>th</sup> month. They were further evaluated for appearance, pH, PS, ZP, %EE and drug content. The obtained values were examined and compiled with fresh formulation output results. All the results were recorded in triplicate (295-299)



# Chapter-5



## Results and discussion

## 5. Results and discussion

### 5.1 Pre formulation studies

#### 5.1.1 Physical examination

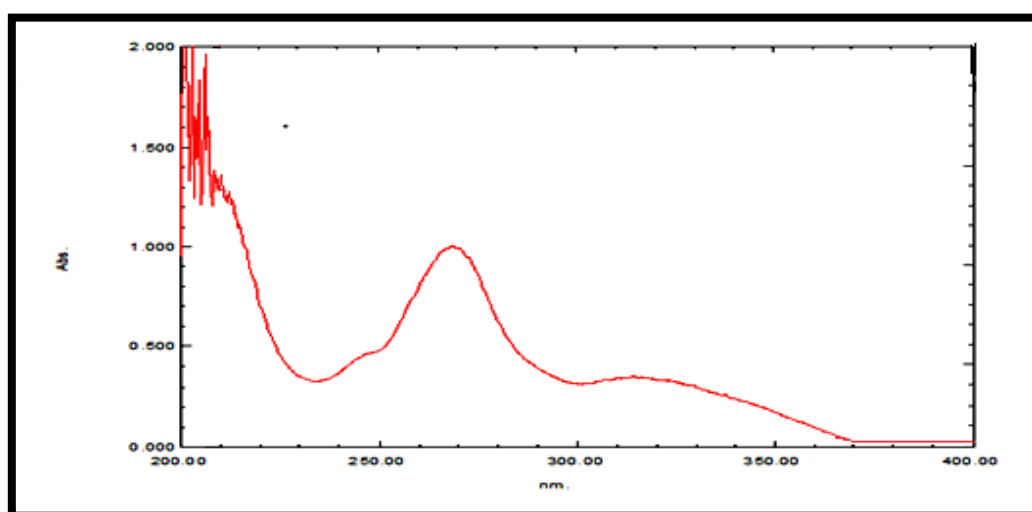
The physical examination of the CS was performed visually after taking on petriplate. The colour of the CS was yellow, as shown in the Figure 10. Its physical nature is solid with characteristic odour. It was having the purity of 95% as per the certificate of analysis.



**Figure 10.** Pure CS

#### 5.1.2 Determination of wavelength maxima ( $\lambda_{\max}$ ) of CS

The wavelength maxima of CS, was found to be 268 nm. It was scanned using UV-spectrophotometer in methanol as blank in the range of 200-400 nm. The UV-spectra of the CS scan is given in the Figure 11



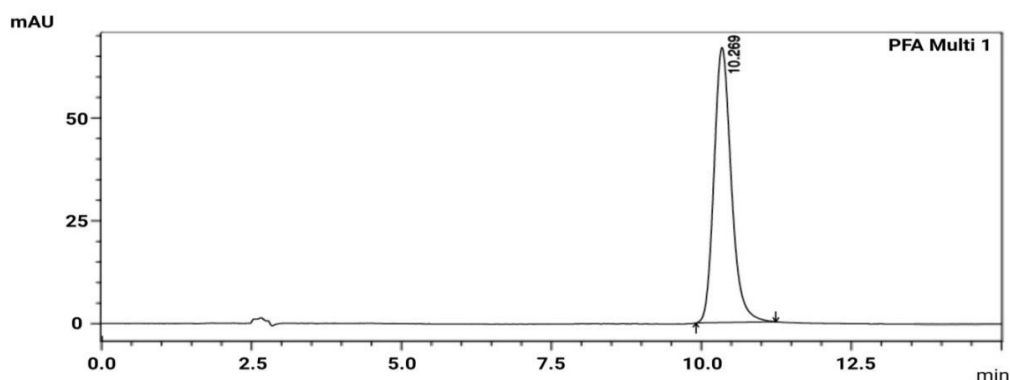
**Figure 11.** UV spectra of CS

### 5.1.3 Method development and validation of CS by RP-HPLC

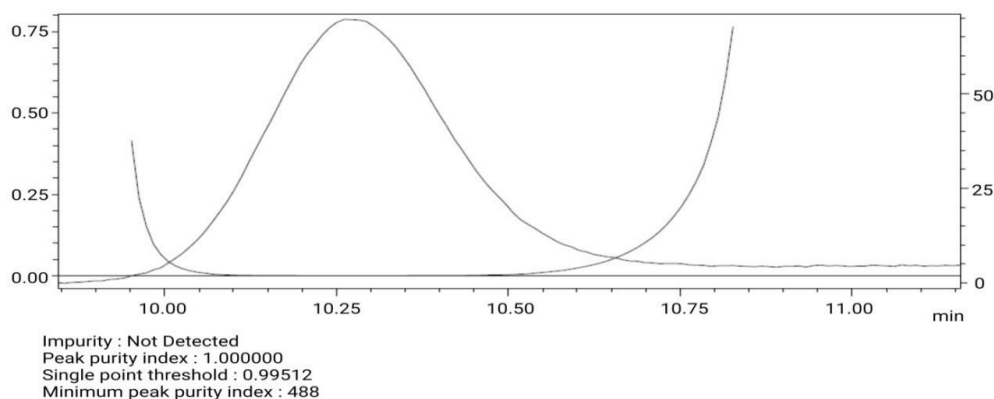
The HPLC method of CS was developed by using the reverse phase HPLC column, by methanol and 0.1% FA as a mobile phase with flow rate of 1 mL/min which was detected at the wavelength of 268 nm.

#### 5.1.3.1 Optimization of mobile phase ratio for estimation of CS

Out of the 50:50 v/v, 60:40 v/v 70:30 v/v ratios 70:30 v/v ratio of methanol and 0.1% FA in water in isocratic elution was shown the better results. An optimized chromatogram of CS shown at 10.29 min as the (Retention time)  $R_t$  with good peak resolution. Hence this ratio was selected for further validation. The developed HPLC chromatogram of CS was shown in Figure 12 and it was symmetric peak. The purity plot of CS shown that there is no presence of the impurity in it and it was shown in Figure 13



**Figure 12.** HPLC chromatogram of CS

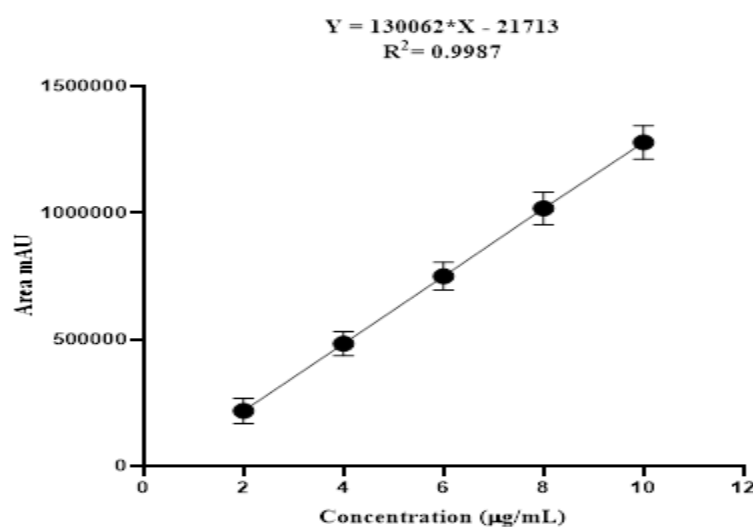


**Figure 13.** Purity plot of CS

### 5.1.3.2 Method validation

#### 5.1.3.2.1 Linearity and Range

The CS calibration curve was constructed by taking the mean area values on the y-axis and concentration values on x-axis. A straight line was obtained from 2-10  $\mu\text{g/mL}$  range and it was found to be linear with a correlation coefficient ( $R^2$ ) value of 0.998 for CS. The plotted calibration curve of CS was shown in **Figure 14**



**Figure 14** Calibration curve of CS

#### 5.1.3.2.2 Accuracy

Accuracy of the CS-HPLC method was accessed by determining the mean % recovery of the “LQC”, “MQC”, and “HQC” solutions in the mobile phase. The data disclosed that for all three levels, the mean percentage recovery in the mobile phase was within the fixed limit (259). The “accuracy” of the developed method was verified by %RSD which was <2%. The accuracy data of CS was shown in Table 22

**Table 22.** Accuracy study of CS

Levels	Concentration of standard solution ( $\mu\text{g/mL}$ )	Actual mean recovery concentration ( $\mu\text{g/mL}$ )	% Recovery	%RSD
LQC	4.8	4.680 $\pm$ 0.24	97.50 $\pm$ 0.34	0.731
MQC	6.0	6.042 $\pm$ 0.21	100.70 $\pm$ 0.21	0.347
HQC	7.2	7.476 $\pm$ 0.34	103.80 $\pm$ 0.28	0.459

#### **5.1.3.2.3 Precision**

The developed method precision was checked by calculating the %RSD for the five injections of the LQC, MQC, and HQC solutions at interday, intraday, and interanalyst levels by using the identical experimental conditions. The observed %RSD was less than 2% for all samples. This clearly indicated that the developed method was suitable and precise. The precision study of CS has shown in Table 23

#### **5.1.3.2.4 Robustness**

Robustness of the established method was checked by varying the ratio of methanol and 0.1% FA (68:32, 70:30 and 72:28), wavelength (266, 268 and 270) flow rate (0.8, 1 and 1.2 mL/min), concentration of FA (0.1%, 0.8% and 0.12% v/v) and lots of column respectively. The observed % RSD was found to be less than 2% for all samples, which showed that the developed method was satisfactory, robust and the responses were not altered by these changes. The robustness study of CS was shown in Table 24

**5.1.3.2.5 “LOD” and “LOQ”:** The “LOD” and “LOQ” of CS was found to be 0.071 µg/mL and 0.217 µg/mL respectively

**5.1.3.2.6 System suitability:** A few of the evaluated system suitability parameters were shown in Table 25 (339)

#### **5.1.4 Specificity**

To find out the potential of separation of method, “specificity” was performed by injecting the excipients alone, placebo as well the formulation to HPLC. It was confirmed from observed peaks that there was no interference of the retention time of CS with adjuvants (336). The obtained results were shown in Figure 15

**Table 23.** Precision study of CS

Parameters	Levels	Conc. (µg/mL)	Area (mAU)					Mean (n = 5)	SD	% RSD
			1	2	3	4	5			
Intraday										
Repeatability	LQC	4.8	593885.	584409	576310	590397	590125	587025.2	6886.48	1.17
	MQC	6.0	784452	780371	795863	776267	796743	786739.2	9202.88	1.16
	HQC	7.2	938545	956202	972514	955051	931322	950726.8	16188.74	1.70
Interday	Intermediate									
Day 1	LQC	4.8	593885	584409	576310	590397	590125	587025.2	6886.48	1.17
	MQC	6.0	784452	780371	795863	776267	796743	786739.2	9202.88	1.16
	HQC	7.2	938545	956202	972514	955051	931322	950726.8	16188.74	1.70
Day 2	LQC	4.8	576363	598485	586063	586496	588530	587187.4	7879.53	1.34
	MQC	6.0	767550	785505	807458	783973	792434	787384	14465.66	1.83
	HQC	7.2	938545	956202	972514	955051	931322	950527.2	16188.74	1.70
Day 3	LQC	4.8	584037	590697	587856	577271	580079	583988.3	5485.72	0.93
	MQC	6.0	799482	786431	765296	757528	774861	776719.6	16693.63	1.14
	HQC	7.2	957259	956580	9545448	951450	954670	934901.4	2262.15	1.81
Inter analyst	Intermediate									
Analyst 1	LQC	4.8	590458	591189	586474	583345	591541	588601.4	3568.31	0.60
	MQC	6.0	788326	788495	793029	796310	777751	788782.3	7012.32	0.88
	HQC	7.2	932932	938404	921290	945284	953212	938224.2	12137.56	0.47
Analyst 2	LQC	4.8	594817	580466	578067	593653	578985	585198.6	8303.65	1.41
	MQC	6.0	785578	761141	794355	794355	794355	785105.2	14383.64	1.76
	HQC	7.2	957459	961896	966437	968506	967490	964357.1	4609.54	0.47
Analyst 3	LQC	4.8	573633	580406	587906	595705	582188	583966.5	8305.32	1.42
	MQC	6.0	797464	782545	789346	757877	789346	783315.3	15170.45	1.93
	HQC	7.2	954576	959407	958224	958034	963856	958819.8	3342.89	0.34

**Table 24.** Robustness study of CS

Variables	Value	Conc. (µg/mL)	Mean peak area	SD	% RSD	Mean Rt (min)	SD	%RSD	
Flow rate (mL/min)	0.8	6	961145.20	17486.89	1.81	10.74	0.17	1.63	
	1.0	6	786739.20	9202.88	1.16	9.32	0.14	1.58	
	1.2	6	652505.40	12964.10	1.98	7.30	0.06	0.86	
Wavelength (nm)	266	6	784677.80	8241.32	1.05	9.26	0.13	1.40	
	268	6	786739.20	9202.88	1.16	9.32	0.14	1.58	
	270	6	756046.60	12798.83	1.69	9.32	0.13	1.41	
Column 1 (34507023)		6	783141.40	41430.25	0.52	7.86	0.07	0.97	
Column 2 (34507034)		6	778232.20	11634.56	1.49	7.54	0.10	1.33	
Column 3 (34505023)		6	397939.90	3471.58	0.08	7.56	0.14	1.87	
Ratios of mobile Phase (methanol: FA)	68:32	6	773000.30	14841.00	1.92	10.64	0.20	1.92	
	70:30	6	786739.20	9202.88	1.16	9.32	0.14	1.58	
	72:28	6	776651.10	7204.00	0.92	7.74	0.76	0.97	
Concentration of FA 0.1% v/v	70:30	6	786739.20	9202.88	1.16	9.32	0.14	1.58	
	0.8% v/v	70:30	6	788282.80	8589.46	1.08	7.90	0.15	1.91
	0.12% v/v	70:30	6	780823.20	14772.55	1.89	8.46	0.12	1.44

**Table 25.** Parameters for evaluation of system suitability

Parameters	Value
HETP	23.380
Theoretical plate	6415.105
Theoretical plate/ meter	42771
Tailing factor	1.239
Peak purity index	0.999

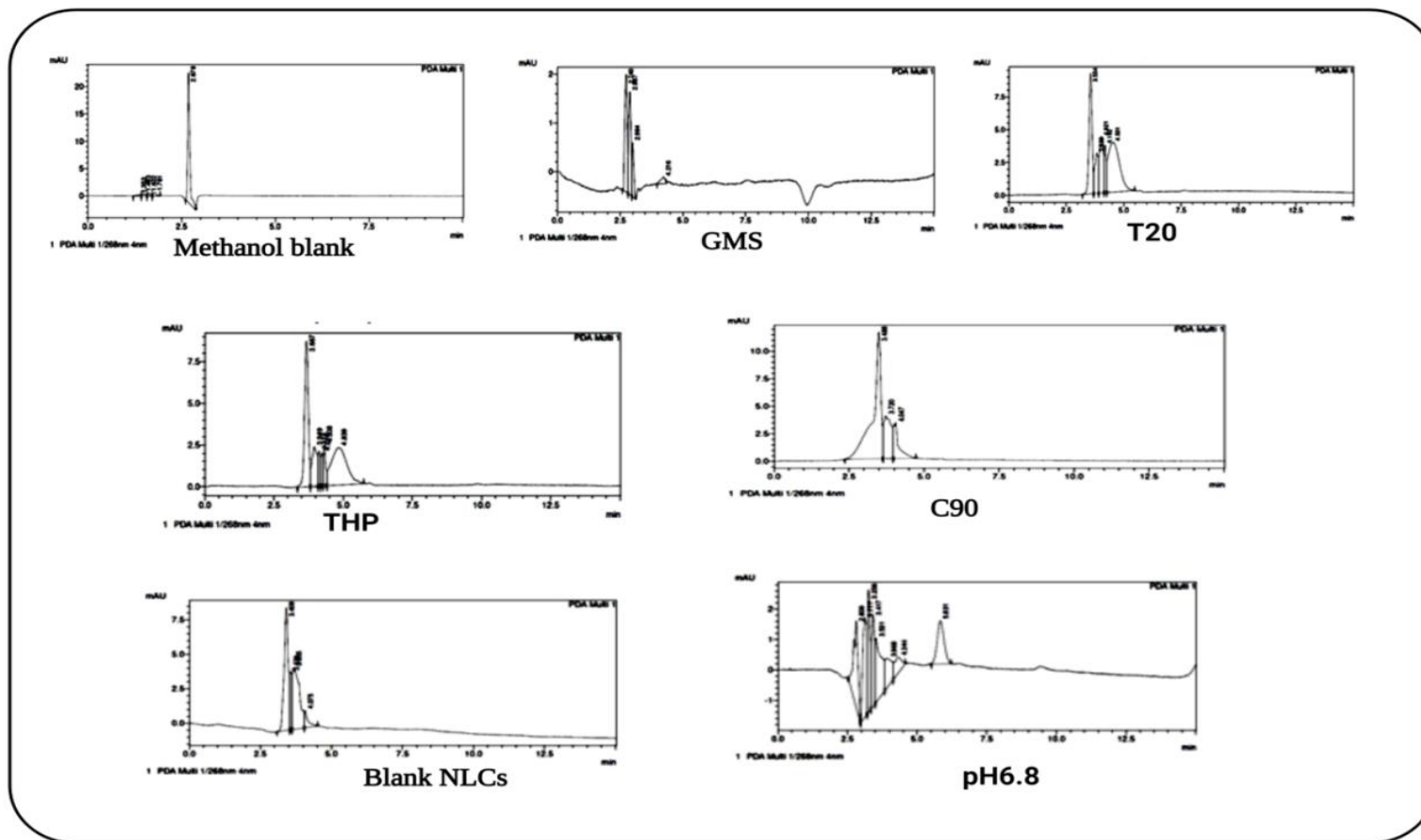
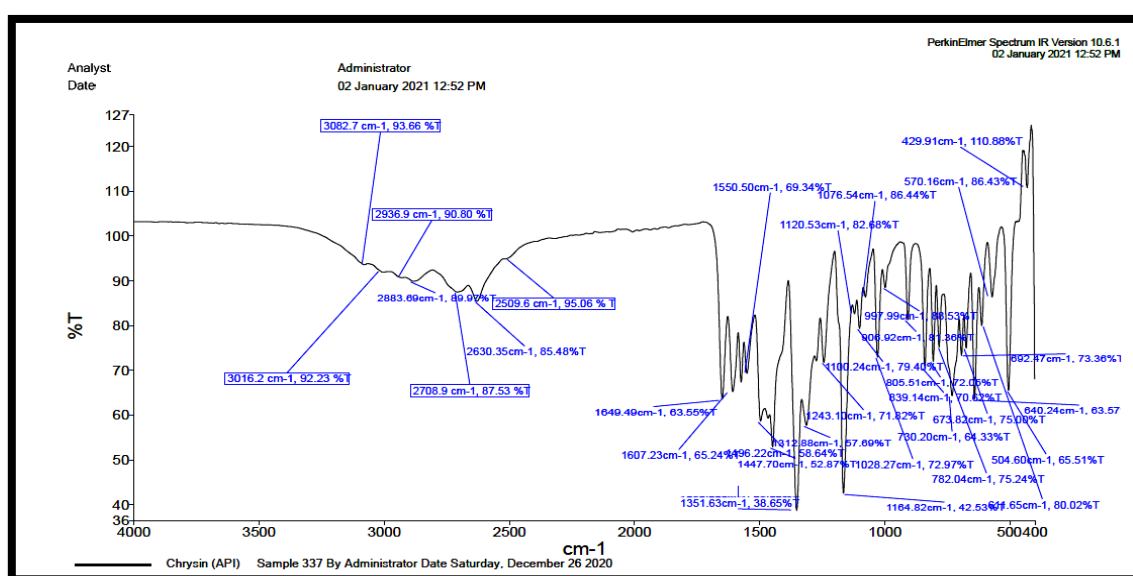


Figure 15. Specificity study of all excipients



## 5.1.5 Characterization of CS

**5.1.5.1 FTIR study of CSCS** is an aromatic molecule having two phenolic groups and one heterocyclic ring. On the basis of vibrational frequency regions obtained from FTIR studies confirmed the presence of all specific functional groups in pure CS was confirmed and represented in the **Table 26**. The FTIR of the CS was shown in **Figure 16**



**Figure 16.** FTIR scan of CS

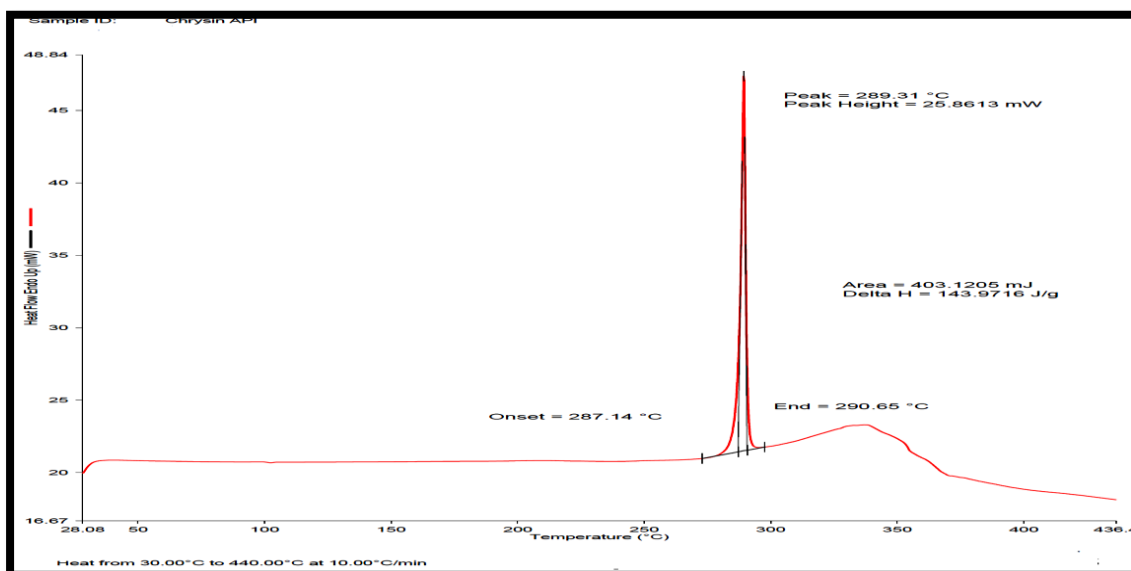
**Table 26.** Frequency region of pure CS

S.No.	Functional group	Observed Wave number (Cm <sup>-1</sup> )	General vibrational frequency regions in flavones (20)
1.	C=O	1649	1650-1600 cm <sup>-1</sup>
2.	OH	3016 & 3082	4000-3000 cm <sup>-1</sup>
3.	C-C-C stretching	1351	—————
4.	C-CO-C Bending	1164	—————
5.	Aromatic group	1550	1600-1500cm <sup>-1</sup>
6.	C=C	1607	1670-1620 cm <sup>-1</sup>

### 5.1.5.2 DSC study of CS

The ‘thermogram’ of CS exhibit melting point at 287.4 °C and no additional peak was detected. This confirmed the presence of pure drug and absence of any polymorphic form. The peak was sharp endothermic which confirmed that CS was crystalline in nature and energy required to melt CS was high (143.97J/g). Almost the same melting point of the drug was reported in literature. The DSC thermogram of the CS was given in the **Figure 17**

The obtained value after performing the melting point of the CS through DSC method was shown in the Table 27



**Figure 17.** Thermogram of CS

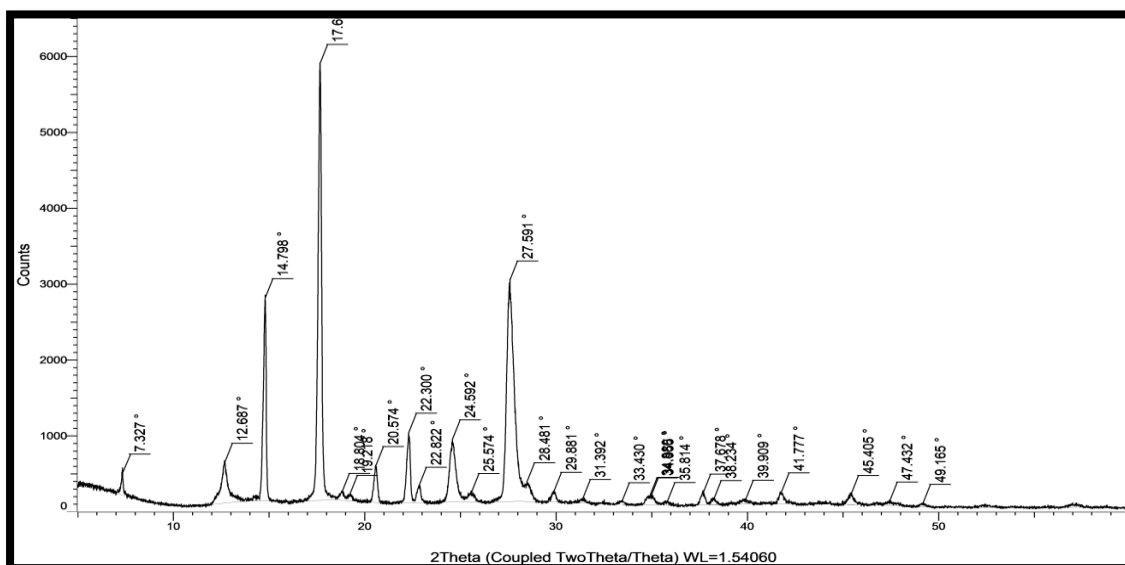
**Table 27.** Melting point of the CS

S.NO.	Observed Endothermic peak	Reported Endothermic peak	References
1.	289.31 <sup>0</sup> C	289.18 <sup>0</sup> C	300-301

### 5.1.5.3 XRD analysis of CS

The physical nature of the CS was obtained by the XRD studies. XRD report shown different sharp peaks at 2 $\theta$  diffraction angle. CS showed many sharp peaks with peak counts of 300, 200, 1100, 400, 900, 200, 200, 400, 300, 100, 150, 200, 500, 600, 2900, 5900 and 3100 at diffraction angles of 18.8, 37.6, 45.4, 19.2, 38.2, 47.4, 22.3, 22.8, 24.5, 22.5, 25.5, 28.4, 29.8, 31.3, 33.4, 34.8, 7.327°, 12.687°, 14.798°, 17.6° and

27.56°. The sharp peak indicates that the drug was crystalline nature (**Figure18**) and the results of powder XRD were found similar with the results of DSC studies.



**Figure 18.** XRD of pure CS

### 5.1.6 Partition co-efficient study of CS

The concentration of the CS was higher in the n-octonol than water, which indicates that CS is lipophilic in nature. Furthermore the Log P value of CS was found close to the reported value in the literature (Puranik & Itadwar, 2020). The obtained Log P value of the CS shown in the Table 28

**Table 28.** Log P value of CS

S.NO.	Observed Log P	Reported Log P	References
1	3.2	3.2	302

### 5.1.7 Preliminary screening of SLs, LLs and SRs based on the solubility of CS

#### 5.1.7.1 SLs

The selection of SLs, LLs, SRs and co-surfactants for the development of CS loaded NLCs was carried out on the basis of a “solubility studies”. Amongst the selected six SLs, the solubility ( $\mu\text{g/gm}$ ) of CS was found as follows in the descending order

GMS ( $12376 \pm 4.58$ ) > Emulsire™ 61WL 2659 pellets ( $11513 \pm 2.21$ ) > Compritol 888 Pellets ( $11191 \pm 1.11$ ) > Geleol ( $9620 \pm 14.42$ ) > Gelucire 43/01 ( $9210 \pm 8.66$ ) > Gelucire 44/14 ( $8290 \pm 2.27$ ) > Palmitic acid ( $3456 \pm 2.08$ ).

It has been observed that CS showed highest solubility in GMS, which may be attributed to the presence of the lower HLB (3.8) (302) and nearer to the log P value of CS (3.2) (301,302). It has been reported that the CS NLCs prepared by using GMS as solid lipid shown higher % EE and sustained release (303,304). Several research studies used GMS as SL for the development of NLCs, for instance Koland and Sindhoor (2021) prepared GMS based apremilast NLCs for psoriasis treatment and the results shown higher permeability and retention of drug upon topical administration (306). Another study performed by Pimpalshend *et al.*, (2018) revealed that stable NLCs were formulated when GMS was used as solid lipid in formulation of betamethasone dipropionate NLCs for topical application (307). NLCs prepared by the GMS as SL have been reported to produce the smaller PS in the range of 57-179 nm and showed better release of drug (308).

#### **5.1.7.2 LLs and SRs**

Amongst the selected nine liquid lipids, the solubility of CS ( $\mu\text{g/mL}$ ) was found as follows in decreasing order capryol 90 (C-90) ( $1179 \pm 1.15$ ) > Soyabean oil ( $1098 \pm 15.71$ ) > Acconon MC8 ( $928.667 \pm 4.50$ ) > Oleic acid ( $450.667 \pm 5.03$ ) > Lauroglycol FCC ( $224.33 \pm 5.68$ ) > Paraffin oil ( $163.53 \pm 2.04$ ) > Migolyol 812 ( $147.43 \pm 2.20$ ) > Labrafac Labraphil WL1349 ( $86.20 \pm 2.03$ ) > Capmul MCM ( $46.667 \pm 2.30$ ). Hence C-90 was selected as LL due to the presence of higher solubility of CS in it. It has been reported that incorporation of liquid lipid into solid lipid improves the %EE and DL due to the development of imperfections in the solid lipid(308).Furthermore LL may reduce the crystalline nature of SL that could help in improving the physical stability of dispersion system. It was confirmed by one of the previous study, reported by Gu.y *et al.*, in 2019, where C-90 was used as LL for development of NLCs loaded with triptolide for rheumatoid arthritis (309). In addition to that C-90 was also able to induce the surface charge on the particles.

It was very essential for getting the stable dispersion system. Abdel-Salam *et al.* in 2017 incorporated the C-90 as liquid lipid for development of diflucortolone valerate NLCs for topical administration which shown higher permeability (310).

Amongst selected six SRs, CS has shown highest solubility in Tween-20 in the following decreasing order in  $\mu\text{g/mL}$ : Tween-20 (T-20) ( $1295 \pm 4.74$ ) > Tween-80 ( $1236 \pm 53.44$ ) > Labrasol ( $1184 \pm 0.57$ ) > Transcutol-HP ( $1087 \pm 16.92$ ) > Poloxamer-407 ( $1037 \pm 2.53$ ) > Poloxamer-188 ( $308 \pm 1.15$ ). The T-20 has been reported to reduce the surface tension between “water and lipids efficiently”, (317) and imparts viscosity for maintaining the stability of the dispersion system. For example, V. M Ghate *et al.*, (2019) employed T-20 as surfactant for development of pentoxifylline NLCs for psoriasis. In addition to that T-20 also provides shielding effect around the particles (319). Furthermore, Transcutol-HP (T-HP) was selected as co surfactant to improve the permeability of the CS across the skin. In addition, T-HP was able to enhance the solubilization and emulsification of surfactant by raising the interfacial fluidity of surfactants as an external surface in the micelles. Further, Transcutol-HP readily permeates through the stratum corneum and by the interaction of the water present in the intercellular path, it will modify the skin permeation of the active drug (312). Makky *et al.*, in 2021 used the T-HP as co-surfactant for the development of caffeine loaded NLCs for treating alopecia (313). From the results of solubilization studies GMS as SL, C-90 as LL, T-20 as SR and T-HP as co-surfactant have been selected. By considering the results of previously reported individual studies, the combination of GMS, C-90, T-20 and T-HP could be able to offer good formulation characteristics.

### **5.1.7.3 Solubility of CS in the phosphate buffers and hydro-alcoholic solutions.**

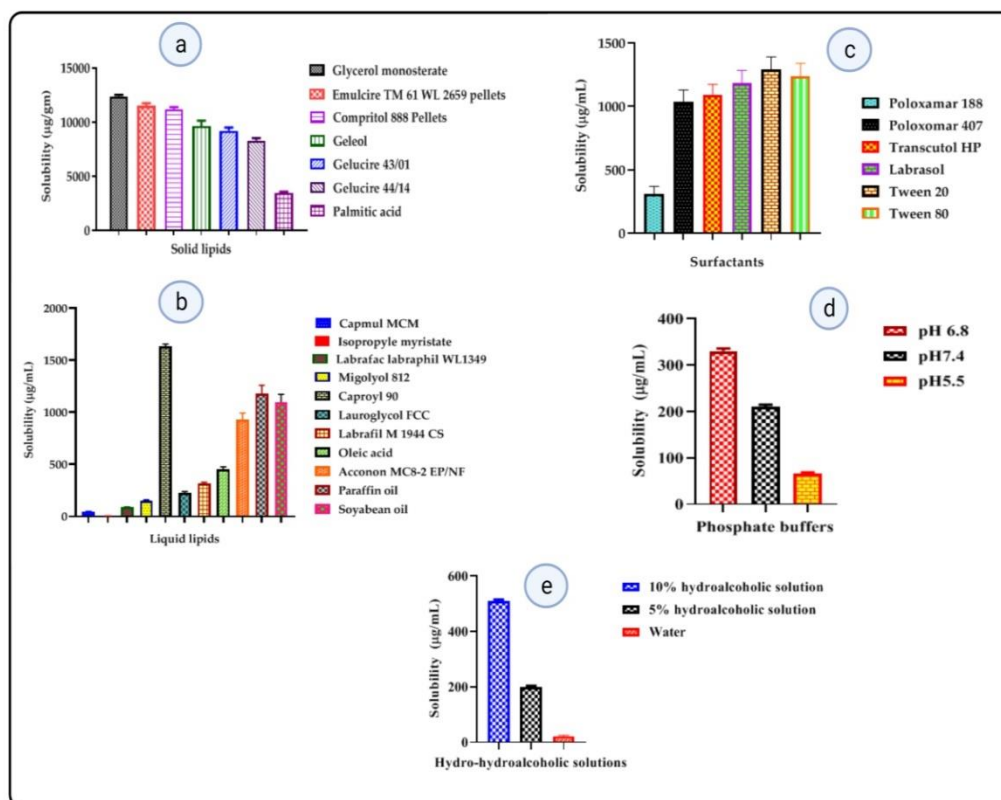
#### **5.1.7.3.1 Phosphate buffers**

Amongst the selected phosphate buffers CS solubility found in decreasing order pH 6.8 ( $330 \pm 5.20$ ) > pH 7.4 ( $210 \pm 3.90$ ) > pH 5.5 ( $65.10 \pm 3.50$ ).

#### **5.1.7.3.2 Hydro and hydroalcoholic solutions**

Amongst the selected hydro and hydroalcoholic solution CS solubility found in decreasing order 10% hydroalcoholic solution ( $510 \pm 5.64$ ) > 5% Hydroalcoholic

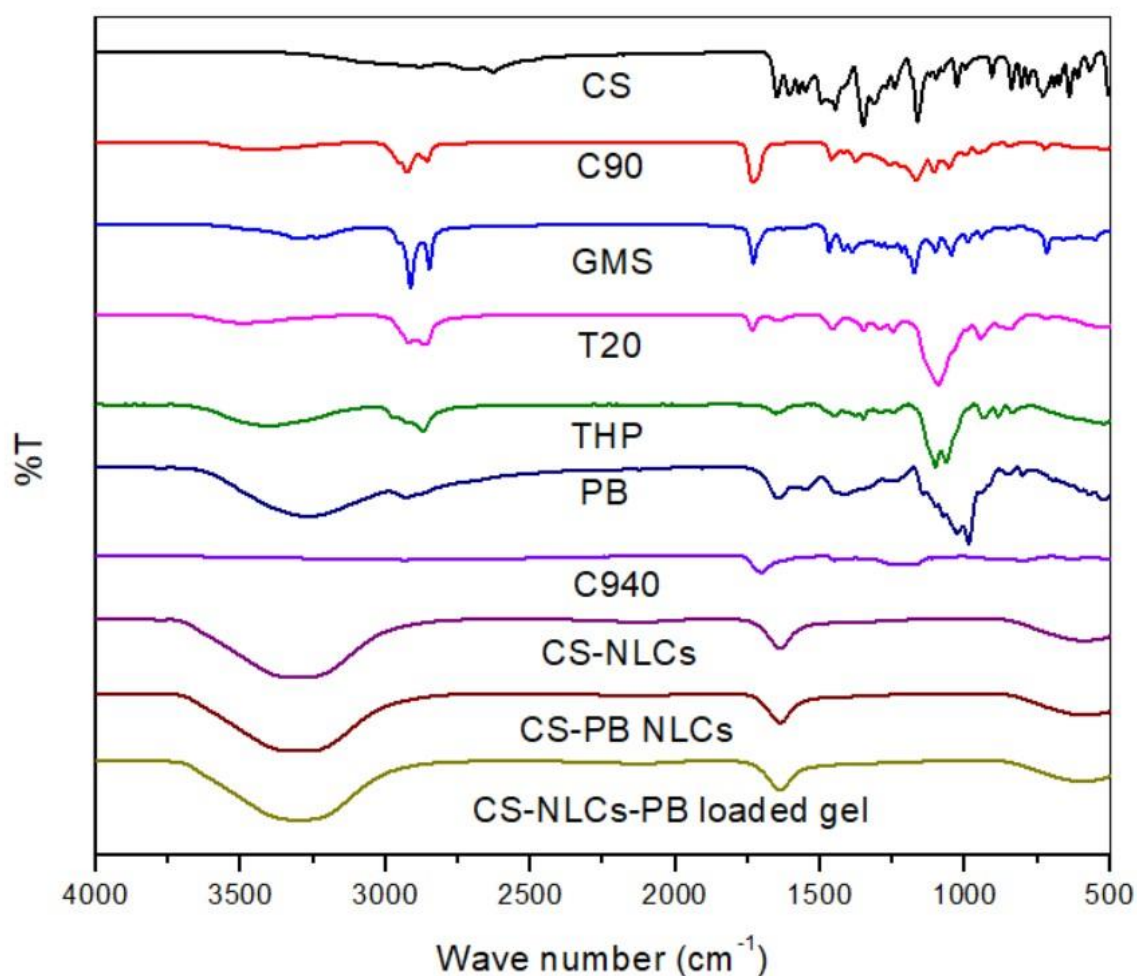
solution ( $200 \pm 2.50$ ) > and in water ( $21.8 \pm 2.40$ ). Graphically represented the amount of CS solubilized in all above mentioned excipients. The results of solubility studies have been depicted in **Figures (19a-19e)**.



**Figure 19 Solubility of the CS in Solid lipid (19a) liquid lipid (19b) surfactant (19c) phosphate buffer (19d) and (19e) hydro-hydro alcoholic solutions**

### 5.1.8 Compatibility studies

As per the results of FTIR it was concluded that there was no physico-chemical interactions between the excipients such as GMS, C90, T20, THP and active agent i.e. CS and PB. The observed results indicated that the drug was incorporated within the lipids, which was confirmed from the FTIR study of the CS-PB NLCs, where we can observe the absence of the functional groups of the CS. It was shown in the Figure 20. Along with there is no additional peak, in the formulation however the intensity of the peak was reduced.



**Figure 20.** Compatibility studies of CS and excipients

## 5.2 Formulation development

### 5.2.1 Construction of TPD

A total of 27 blank nano emulsions (Figure 21) were prepared in the ratio of 1:1, 2:1 and 3:1 of Lmix and Smix as shown in Table 29. Out of the 27 formulations, 6 formulations were transparent as per the visual observation and transparency test. Furthermore, it was confirmed from the TPD (Figure 22) that the formulations F1, F2, F10, F11, F12 and F19 were transparent and formed the nano emulsions. The transparent formulations were selected for further study and they were converted into CS loaded NLCs. The CS loaded NLCs were evaluated for PS, ZP, PDI, %EE and %DL (314). It was shown in the Figure 23

### 5.2.2 Development of CS loaded NLCs

The CS loaded NLCs were developed by the method discussed in the section 4.2.1.6.2. The developed CS loaded NLCs were shown in the **Figure 23**. The results of PS and ZP of all six, CS loaded NLCs showed less than 200 nm and PDI less than 0.3, which indicated the formulation are in nano size and are well dispersed respectively. The ZP was found in the range of -12.7 mV to -16.5 mV, which indicated the presence of the repulsive forces between the particles and NLCs were stable. It may be due to the non-ionic property of surfactant. The % EE was found between the 80 to 98% which indicated the better entrapment efficiency of lipids for CS and optimum loading (8.7-9.4%). The trial results were given in the **Table 30** Hence, from these three levels (low, medium, high) of SL, LL, SRs and co-surfactant were selected and further study was carried out by DOE. To develop the optimized CS loaded NLCs **BBB** design was used



**Table 29.** Aqueous titration method

Batch	Lmix GMS: C-90 mg	Smix(T- 20:T-HP) mg									Water end point value (mL)	
		9	8	7	6	5	4	3	2	1		
		1:1	1:1	1:1	1:1	1:1	1:1	1:1	1:1	1:1		
F1	50:50	450:450										100
F2	100:100		400:400									100
F3	150:150			350:350								4
F4	200:200				300:300							0.8
F5	250:250					250:250						1
F6	300:300						200:200					1.5
F7	350:350							150:150				0.5
F8	400:400								100:100			0.3
F9	450:450									50:50		0.2
		2:1	2:1	2:1	2:1	2:1	2:1	2:1	2:1	2:1		
F10	66:33	600:300										94
F11	133:66		533:266									72
F12	200:100			466:233								60
F13	266:133				400:200							1
F14	353:166					353:166						1
F15	400:200						266:133					1
F16	466:233							200:100				1.5
F17	533:266								133:66			0.5

F18	600:300									66:33	0.5
		3:1	3:1	3:1	3:1	3:1	3:1	3:1	3:1	3:1	
F19	75:25	675:225									100
F20	150:50		600:200								100
F21	225:75			525:175							4.5
F22	300:100				450:150						1.5
F23	375:125					375:125					2.0
F24	450:150						300:100				0.6
F25	525:175							225:75			0.7
F26	600:200								150:50		1.2
F27	675:225									75:25	0.5

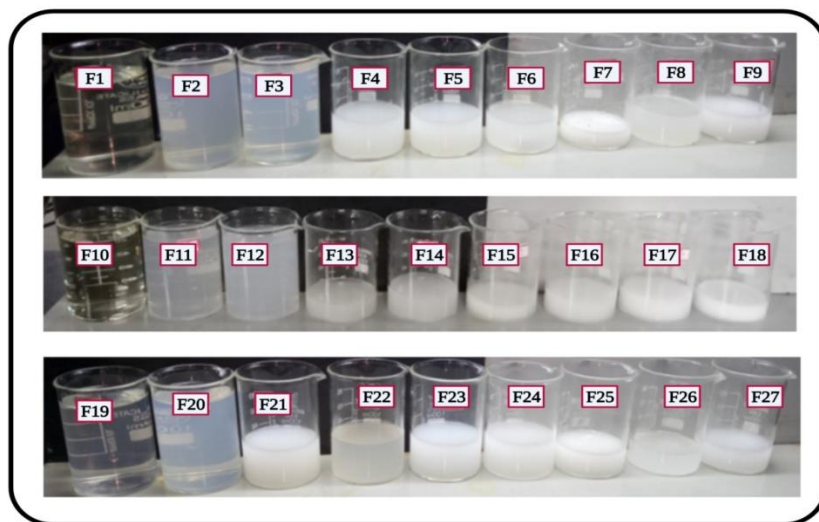


Figure 21. Blank nano emulsions

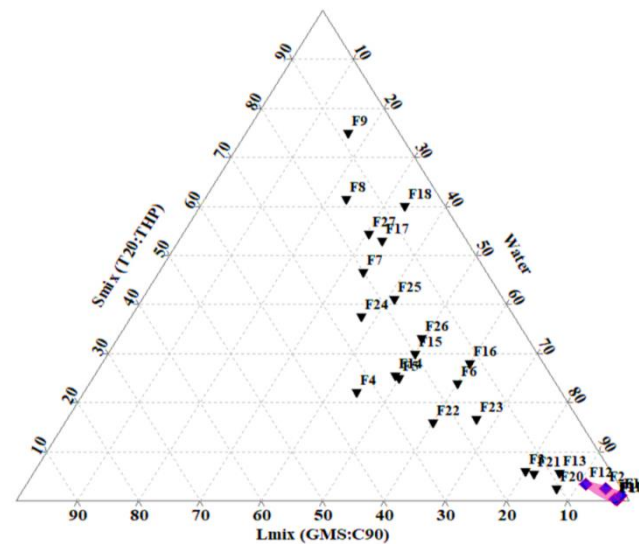


Figure 22 Ternary phase diagram

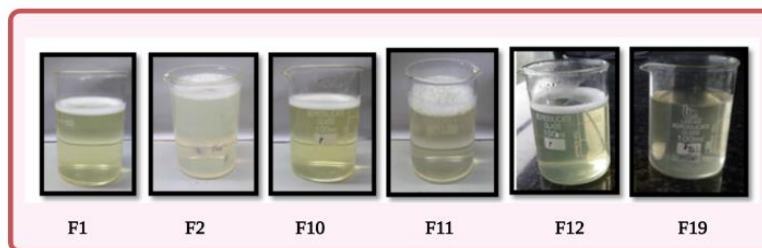


Figure 23. CS loaded NLCs

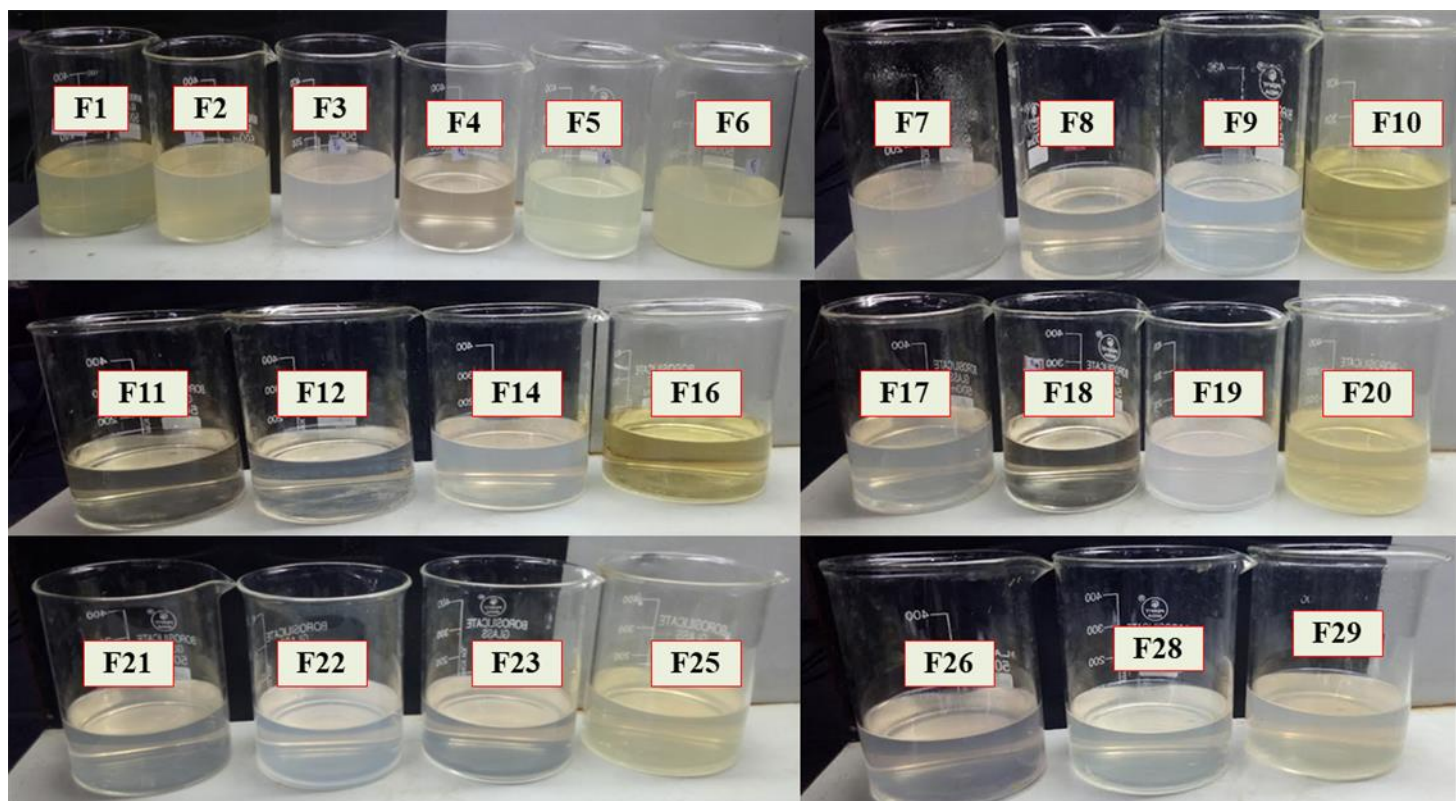
**Table 30.** Characterization of CS loaded NLCs (n=3)

Batch	Solid lipid	Liquid lipid	Surfactant	Co -surfactant	Ratios	PS (nm)	ZP (mV)	PDI	EE %	DL %
	GMS (mg)	C-90 (mg)	T-20 (mg)	T-HP (mg)						
F1	50	50	450	450	1:1	167 ± 64.99	-12.7± 1.22	0.101± 6.49	98 ± 1.26	8.7 ± 0.15
F2	100	100	400	400	1:1	133 ± 23.00	-16.5± 2.22	0.293 ± 2.30	98 ± 1.55	9.0 ± 0.89
F10	66	33	600	300	2:1	77 ± 13.88	-15.0± 3.22	0.271 ± 1.38	96 ± 0.66	9.0 ± 0.82
F11	133	66	533	266	2:1	147 ± 10.22	-15.5± 7.66	0.294 ± 1.02	90 ± 1.55	9.4 ± 0.78
F12	200	100	466	233	2:1	102 ± 11.25	-12.7± 8.99	0.294 ± 1.12	80 ± 0.89	9.0 ± 0.77
F19	75	25	675	225	3:1	82 ± 8.99	-15.6± 8.09	0.217 ± 8.99	98 ± 0.78	9.4± 0.77

### 5.2.3 Optimization of CS loaded NLCs by BBD design

The total 29 formulations were carried out with BBD which were shown in the Figure 24. To investigate the effect of adjuvant used to formulate NLCs (SL (A), LL (B), SRs (C) and co-surfactant (D)) on ZP, % EE and PS. The data of responses for all the experiments runs designed by BBD is depicted in Table.31 The magnitude and significance impact of main variables and their interactions were determined by analysis of variance (ANOVA).(316-317). The counter plots for independent factors were generated by the use of the obtained polynomial equations (PE) from BBD. The adequacy of the model was confirmed by the use of ANOVA i.e ( $P < 0.05$ ). The summary of results for all responses was shown in Table.32

The polynomial coded equations explained that SL, LL, SRs and co-surfactant have significant effects on % EE, ZP and PS. All the equations were shown in the **Table 33**. for PS, ZP and % EE respectively were quadratic. The coded factors determined by design expert software are shown in below equations and final mathematical model was determined. The counter plots for various independent factors were plotted by these equations (315). The positive and negative sign of PE indicates the synergistic and antagonistic effects against the responses respectively. The PE indicated that the PS was decreased with increase in factor A and D, where as it is increased with increase in factor B and C. ZP was decreased with increase in factor A, B and C, while it is increased with increase in factor A and decreased with increase of factor B, C and D. The perturbation plots were also plotted to understand the effect of factors on the responses. The straight and flat lines of these plots indicated no impact of factor on responses where as a significant impact was indicated by steep and bent curves (316,317).



**Figure 24.** CS loaded NLCs as per BBD

**Table 31.** The data of responses for all the experimental runs designed by BBD for CS loaded NLCs

Formulations	Independent variables				Responses		
	GMS (mg)	C-90 (mg)	T-20 (mg)	T-HP (mg)	PS (nm)	ZP (mV)	%EE
F1	125.0	62.5	400.0	450.0	133.0	-16.8	96.0
F2	125.0	62.5	550.0	337.5	169.0	-19.9	95.0
F3	200.0	62.5	550.0	225.0	136.0	-19.2	94.0
F4	50.0	62.5	550.0	450.0	199.0	-18.9	93.0
F5	200.0	62.5	700.0	337.5	98.6	-20.3	96.0
F6	125.0	62.5	400.0	225.0	91.0	-21.2	92.0
F7	200.0	62.5	400.0	337.5	58.0	-18.6	98.0
F8	125.0	25.0	550.0	225.0	151.0	-19.4	95.0
F9	200.0	62.5	550.0	450.0	100.0	-16.7	93.0
F10	50.0	100.0	550.0	337.5	198.0	-20.1	89.0
F11	50.0	25.0	550.0	337.5	140.0	-18.0	97.0
F12	50.0	62.5	400.0	337.5	173.0	-18.7	97.0
F13	125.0	62.5	550.0	337.5	169.0	-19.9	94.0
F14	125.0	100.0	700.0	337.5	110.0	-22.3	89.0
F15	125.0	62.5	550.0	337.5	169.0	-19.9	96.0
F16	50.0	62.5	700.0	337.5	137.0	-19.8	96.0
F17	125.0	100.0	550.0	450.0	169.0	-19.6	88.0
F18	50.0	62.5	550.0	225.0	186.0	-17.6	93.0
F19	200.0	25.0	550.0	337.5	91.0	-19.9	95.0
F20	125.0	62.5	700.0	450.0	87.0	-21.4	86.0
F21	125.0	62.5	700.0	225.0	151.0	-18.6	95.0
F22	125.0	25.0	400.0	337.5	74.0	-20.3	94.0
F23	125.0	100.0	400.0	337.5	100.0	-19.7	96.0
F24	125.0	62.5	550.0	337.5	172.0	-19.9	95.0
F25	125.0	100.0	550.0	225.0	117.0	-20.6	85.0
F26	125.0	25.0	700.0	337.5	73.0	-20.3	96.0
F27	125.0	62.5	550.0	550.0	169.0	-19.0	94.0
F28	125.0	25.0	550.0	550.0	79.0	-19.5	89.0
F29	200.0	100.0	550.0	550.0	90.0	-19.3	96.0

**Table 32.** Summary of results for all responses

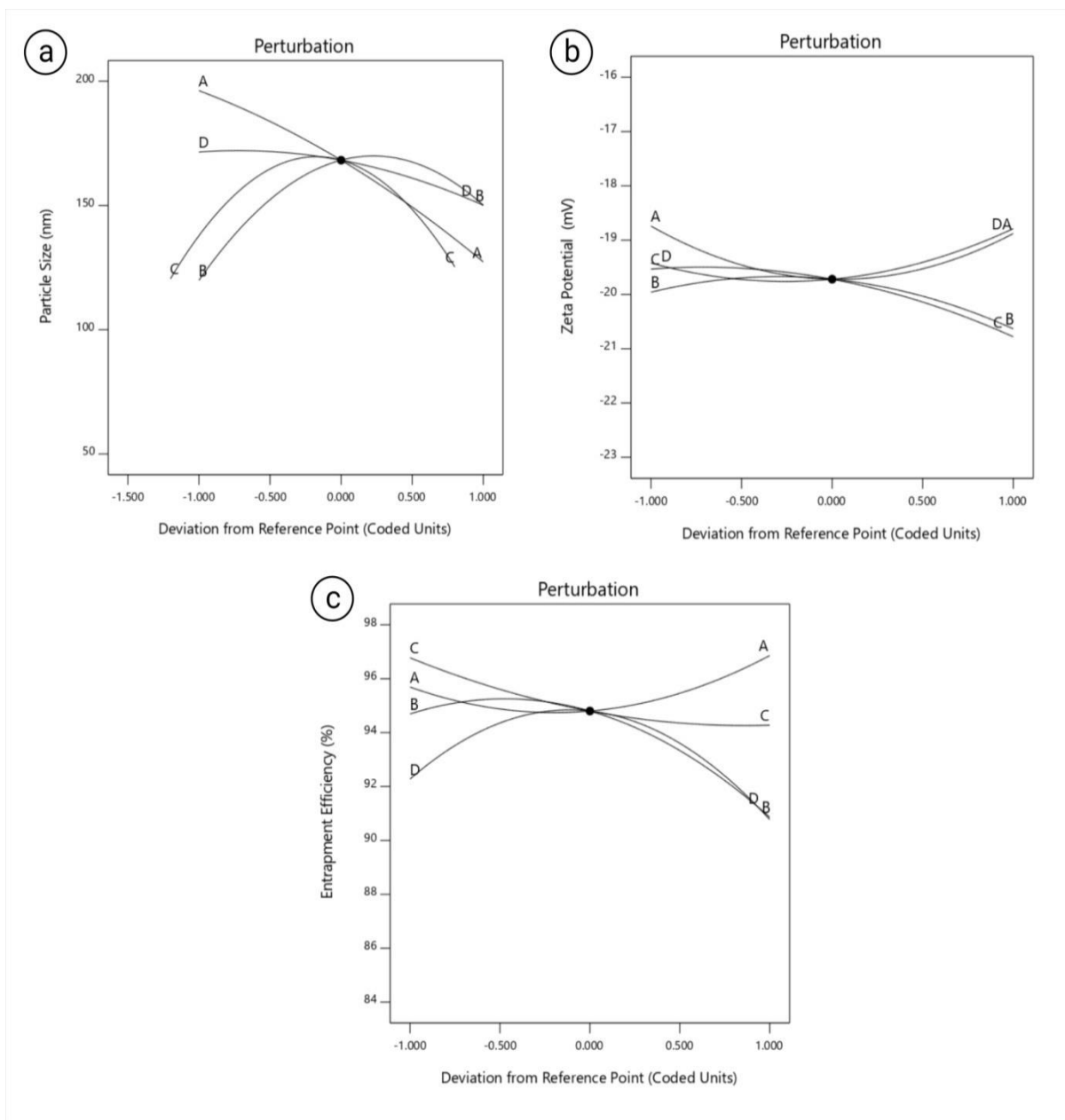
Response	Sequential p-value	Adjusted R <sup>2</sup>	Predicted R <sup>2</sup>	Suggested model	Sequential model sum of squares P- Value
Particle size	< 0.0001	0.9886	0.9967	Quadratic	<0.0001
ZP	< 0.0001	0.9376	0.8841	Quadratic	<0.0001
% EE	<0.0001	0.9064	0.7667	Quadratic	<0.0001

**Table 33.** Polynomial equations for PS, ZP and % EE

Factors	Intercept	PS	ZP	% EE
	$\beta_0$	+169.60	-19.72	+94.80
*A	$\beta_1$	-38.27	-0.06	+0.5833
*B	$\beta_2$	+14.59	-0.3367	-1.92
*C	$\beta_3$	+2.38	-0.6233	-1.25
*D	$\beta_4$	+5.42	+0.3158	-0.75
*AB	$\beta_5$	-14.98	+0.6550	+2.25
*AC	$\beta_6$	+19.15	-0.1300	-0.25
*AD	$\beta_7$	-12.05	+0.9625	-0.25
*BC	$\beta_8$	+2.59	-0.6500	-2.25
*BD	$\beta_9$	+31.12	+0.2650	+2.25
*CD	$\beta_{10}$	-26.44	-1.80	-3.25
*A <sup>2</sup>	$\beta_{11}$	-6.47	+0.9100	+1.48
*B <sup>2</sup>	$\beta_{12}$	-33.22	-0.5763	-2.02
*C <sup>2</sup>	$\beta_{13}$	-46.69	-0.4362	+0.72
*D <sup>2</sup>	$\beta_{14}$	-7.41	+0.615	-3.28

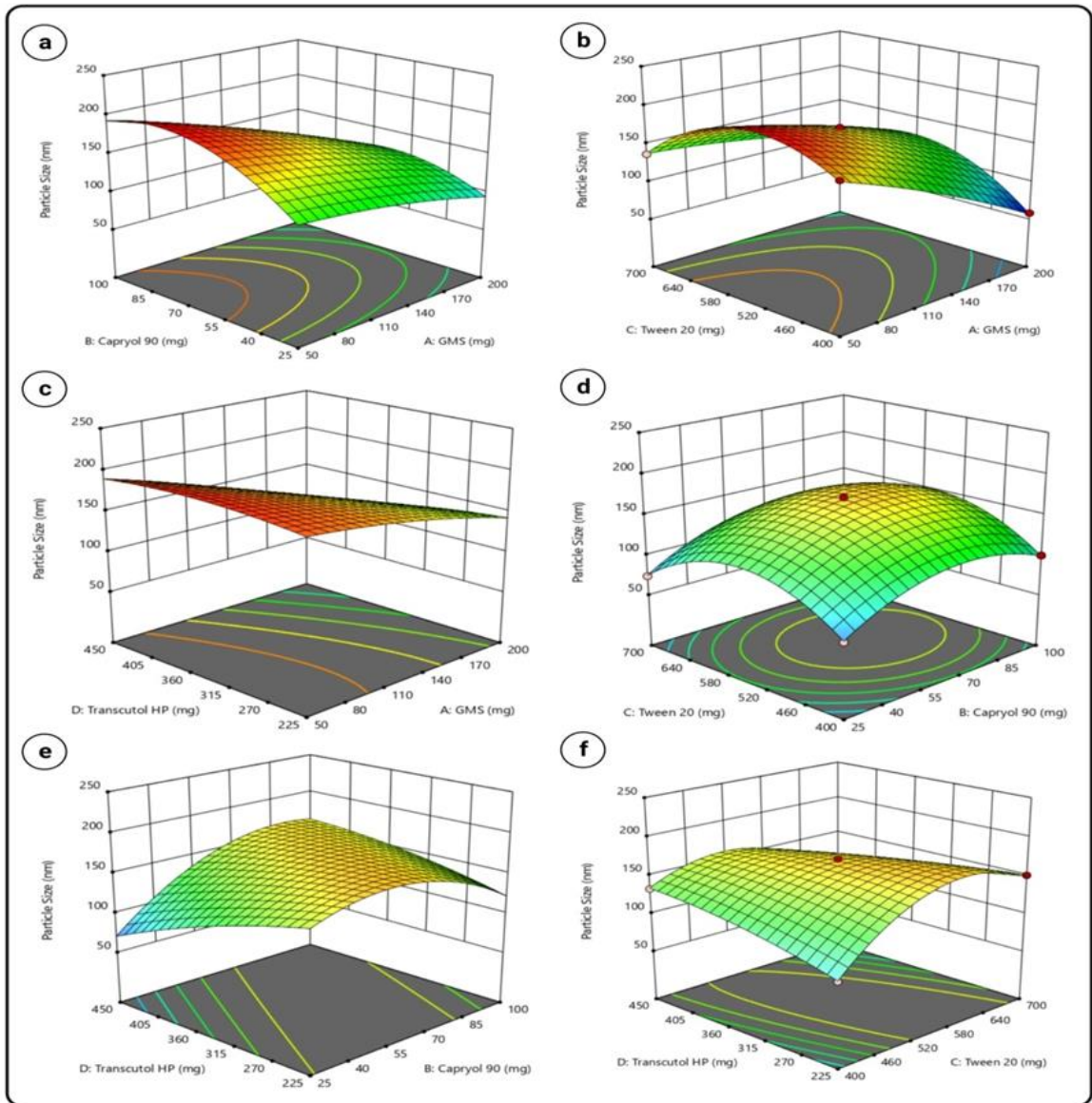
The perturbation plot shown in Figure 25 a, represented that factor “C” was “more dominant than other factor A, B and D on PS”. “Similarly, perturbation plot shown in Figure 25 b, showed that factor “B” has “more dominant effect than other A, C and D factors on ZP” (317). Similarly, “perturbation plot shown in Figure 25 c indicated that factor “D” had “more dominant effect on % EE” than the other factors A, B and C for CS loaded NLCs”(317). The obtained polynomial equations helped in establishment of 3D response surface plots (3D-RSP) as shown in Figure.26 (a-f). The 3D-RSP (Figure. 26 a) indicated a decrease in “PS” “with increase in factor A and decrease in factor B” (317). Figure.26 b indicated, “increase in factor A and C resulted in decreased PS”. Similarly, Figure.26 c indicated that “increase in factor A and D resulted in decreased PS”. In the same manner Figure.26 d revealed that “decrease in factor B and increase of factor C resulted in reduction of the PS”.



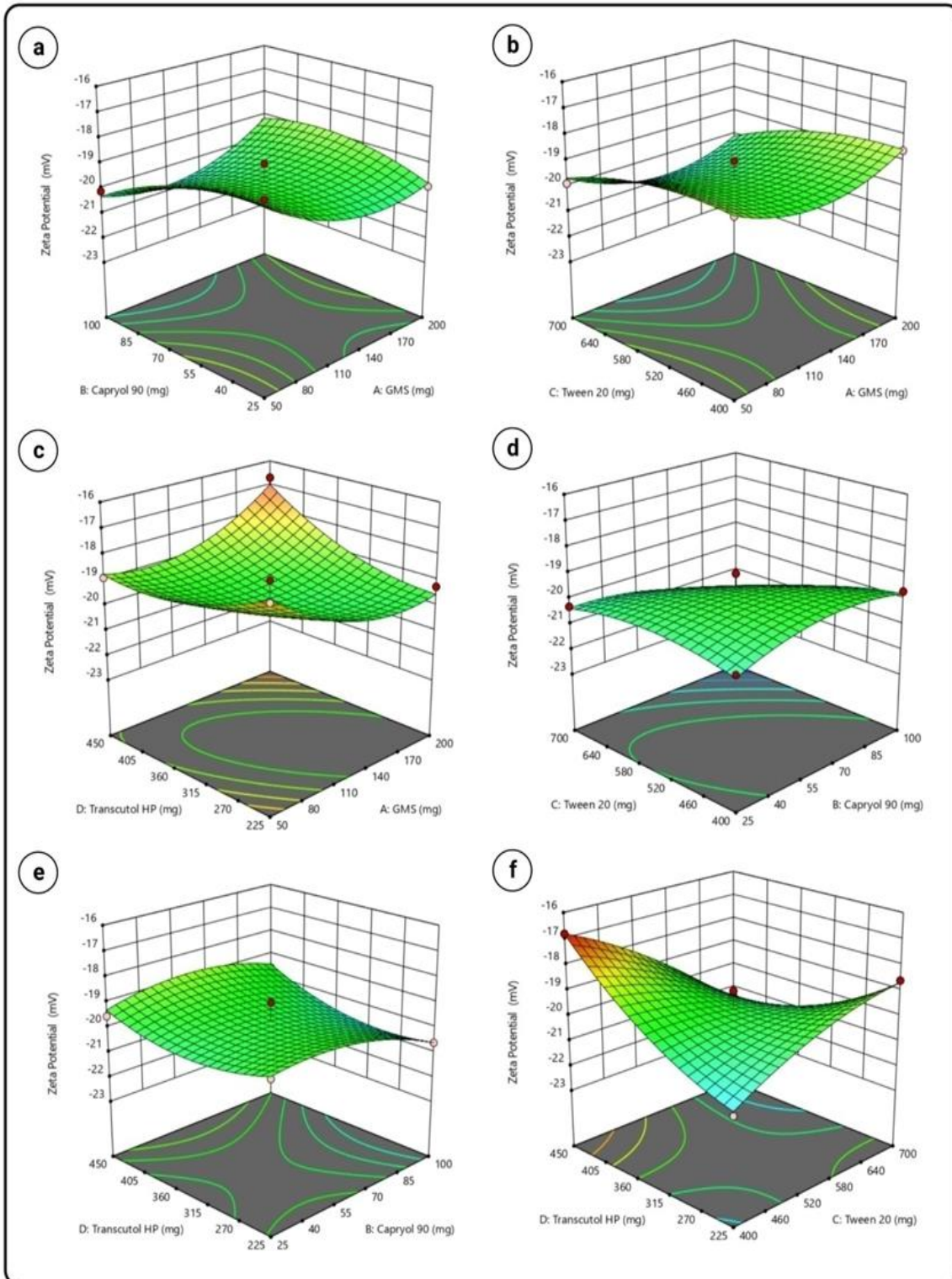


**Figure 25.** (a-c) “Perturbation plots indicating the impact of factors A, B, C and D on responses a.PS, b. ZP and c. % EE” (317)

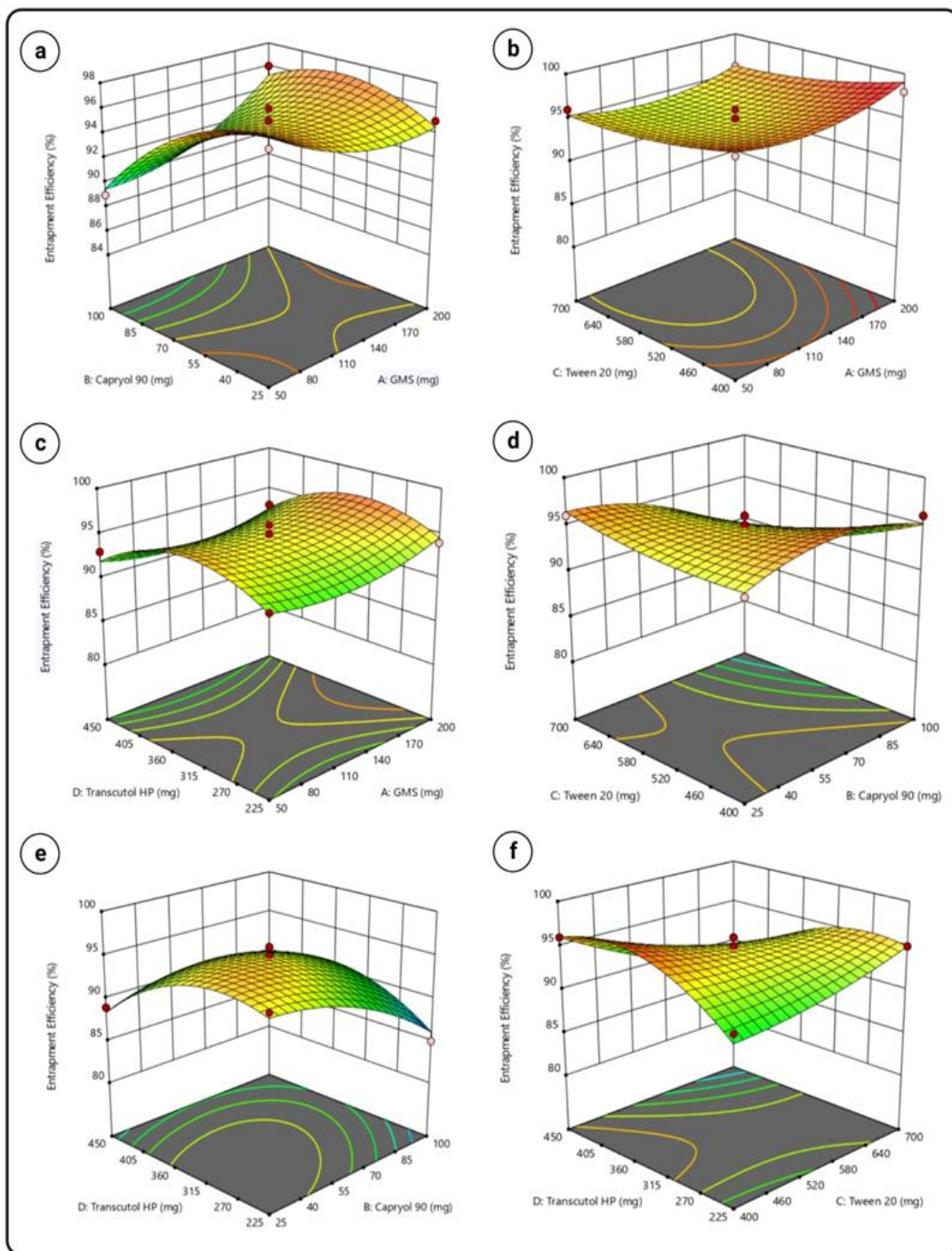
Correspondingly Figure. 26 e indicated that “decrease in factor B and increase in factor D caused reduction in PS”. Identically, Figure 26 f represented that “increase in factor C and D levels caused reduction in PS”. Figure 27 (a-f) the 3D RSP showing the effect of factor A, B, C and D on ZP. Figure.27 a indicated that “increase in factor A and B shifted the ZP towards negative side”. Figure.27 b represented “increase in factor A and C shifted the negative value of ZP towards higher side”. (317).The Figure.27 c indicated that higher the value of factor A and D more the negative value of ZP. Similarly, Figure.27 d represented as the increase in of factor B and C resulted in higher negative sign of ZP. Similarly Figure 27 e showed that the increase in factor B and D shifted the ZP value towards more negative side. Similarly “increase in factor C and D also lead to shift of ZP” (Figure 27 f). The 3D RSPs for % EE were shown in the Figure 28 (a-f). Figure 28 a “increased % EE was observed with increase in factor A and B”. Similarly, “increase in factor A and C resulted in increased the % EE” (Figure.28 b). Similarly, “increase in factor A, D (Figure. 28 c), B, C (Figure 28 d), B, D (Figure 28 e) C and D (Figure.28 f) resulted in increased % EE” (317)



**Figure 26 (a-f).** 3D RSP showing the effect of factor A, B, C and D on PS



**Figure 27 (a-f).**3D RSP showing the effect of factor A, B, C and D on ZP



**Figure 28(a-f).**3D RSP showing the effect of factor A, B, C and D on %EE

### 5.2.3.1 Preparation of optimized CS loaded NLCs

#### 5.2.3.1.1 Graphical optimization of CS loaded NLCs

As per the BBD SL, LL, SR and co surfactant optimized values were found to be 199.99 mg, 33.92 mg, 700 mg and 376.869 mg respectively. It was given in the overlay plot, which showed the “predicted values and responses for formulation of NLCs” (317) (Figure 29)

The “BBD design suggested that using above mentioned values, CS loaded NLCs can be formulated having the PS in the range of 50.32 to 57.98 nm, ZP in the range of -21.09 to -19.55 mV and % EE in the range of 91 to 96” (317). The optimized CS loaded NLCs were developed as procedure mentioned in the section 4.2.1.6.2. The developed optimized CS loaded NLCs were shown in the **Figure 30** The reproducibility of the developed NLCs containing CS was verified for PS, ZP and % EE (n=3).

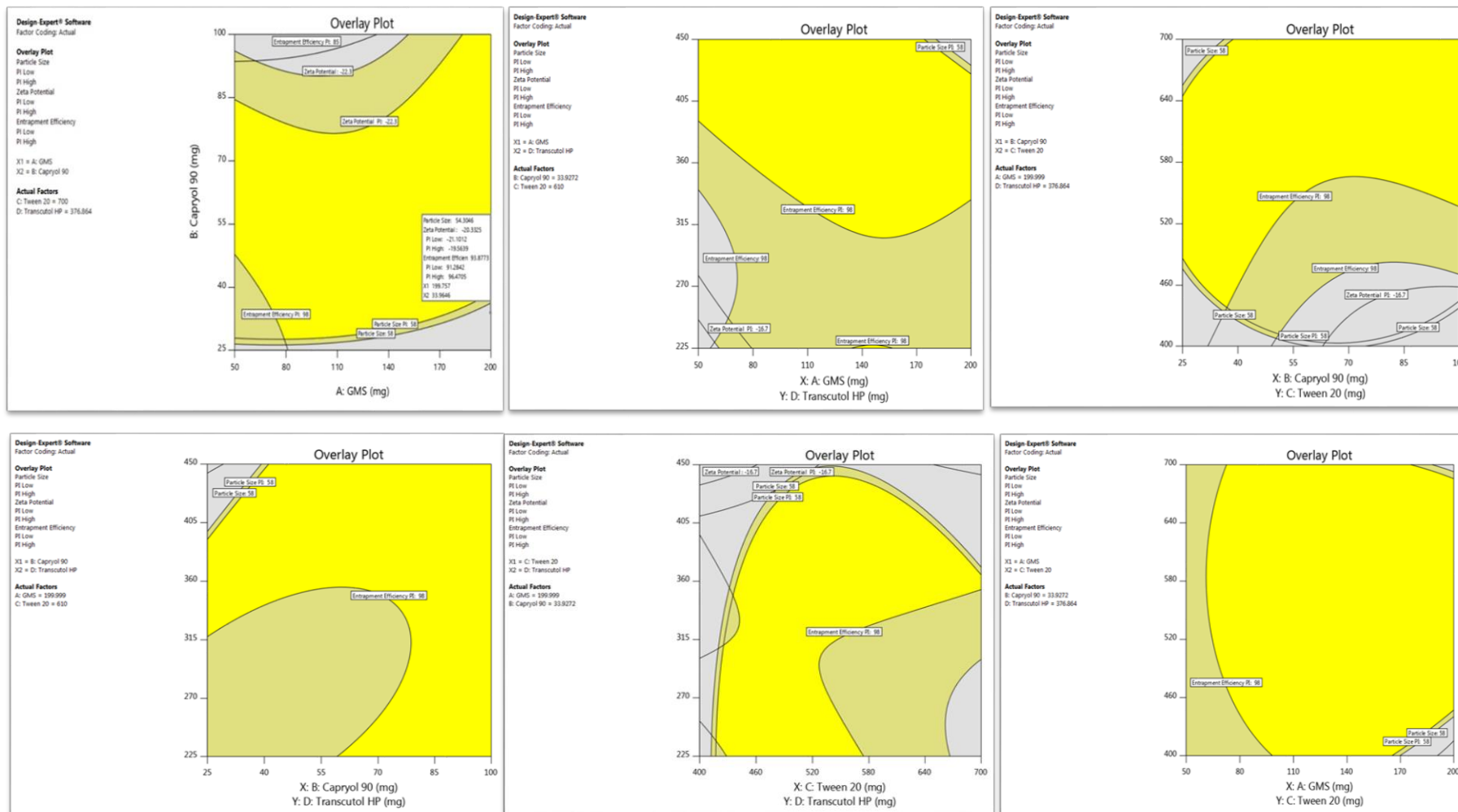
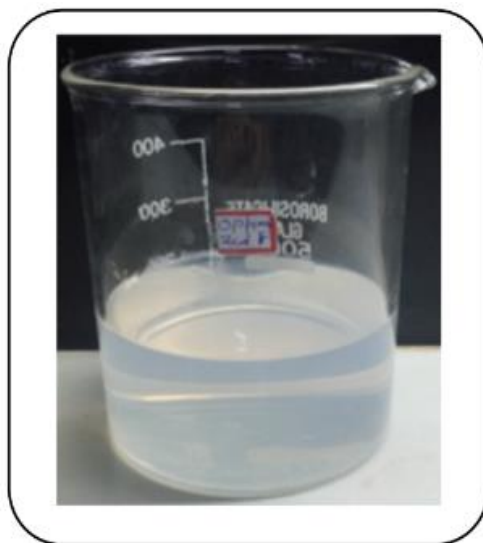


Figure 29. Overlay plot for formulation of CS loaded NLCs showing the predicted values and responses

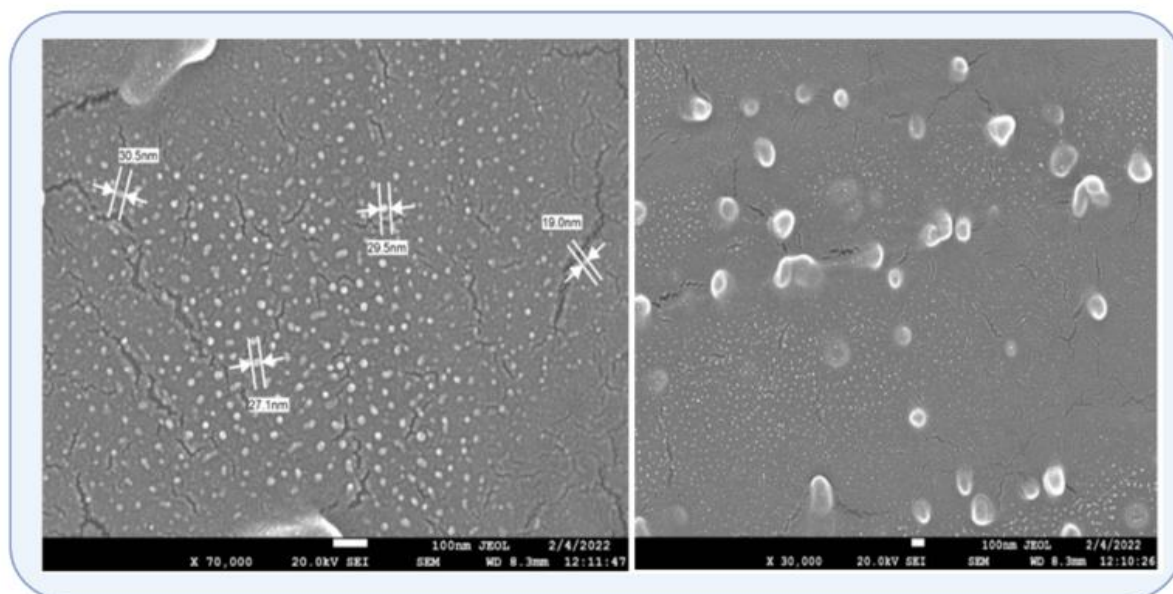


**Figure 30.** Optimized CS loaded NLCs

### 5.3 Characterization of developed *Chrysin* loaded nanostructured lipid carriers

#### 5.3.1 Morphological study of CS loaded NLCs

The morphological structure of CS loaded NLCs was found to be smooth spherical in shape with nano size range and without aggregation of particles. The results were shown in the Figure 31

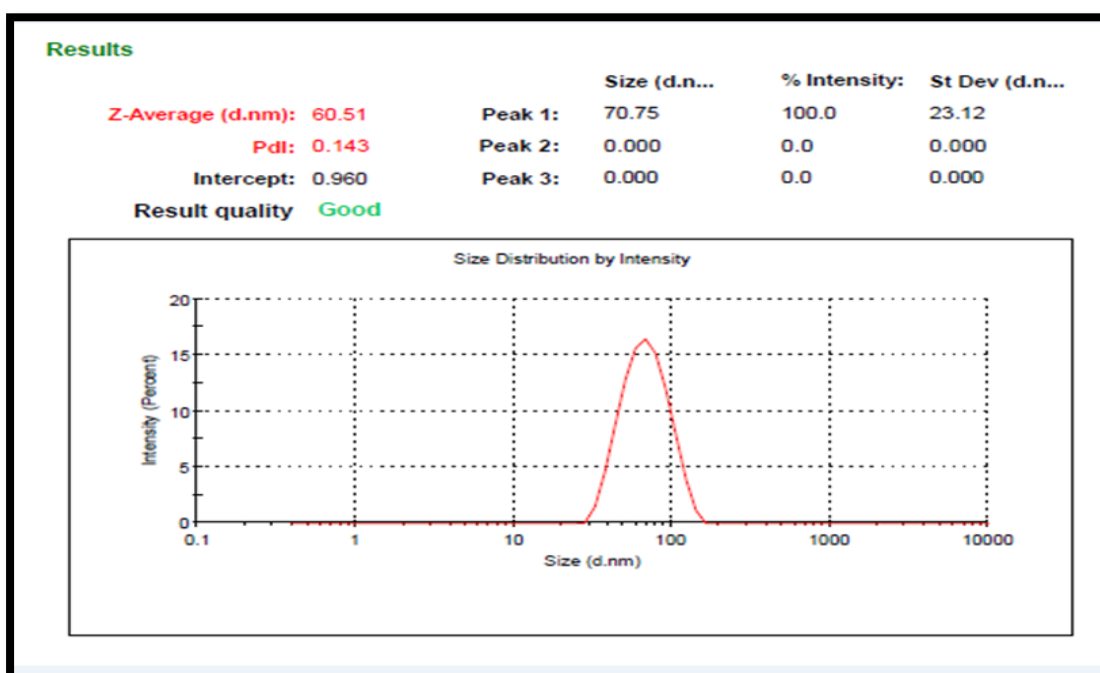


**Figure 31.** FESEM of CS loaded NLCs



### 5.3.2 PS and PDI of optimized CS loaded NLCs

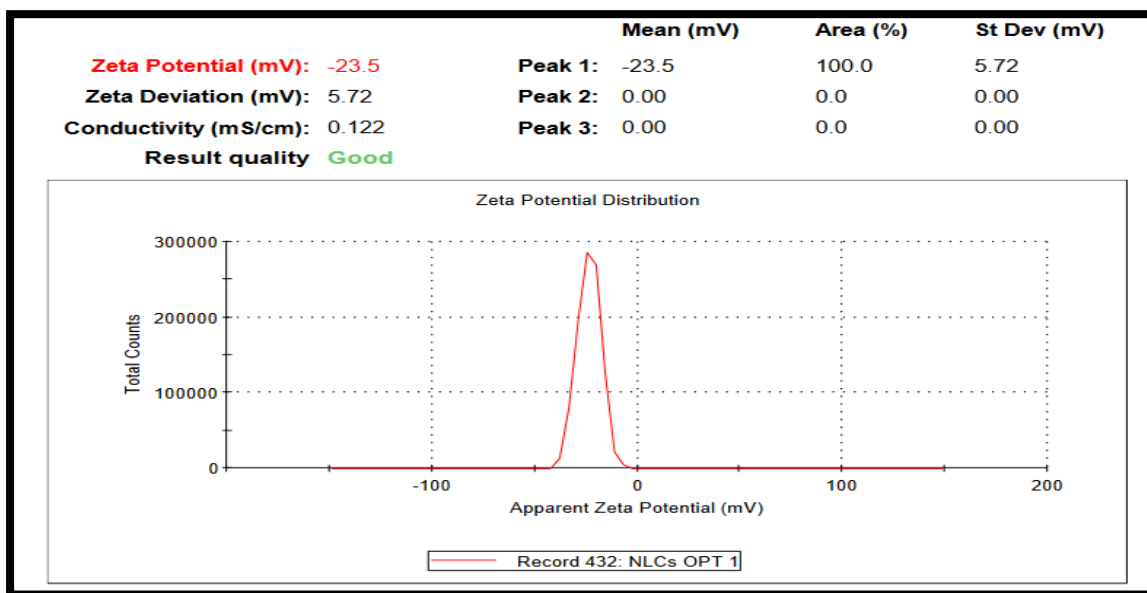
The PS of the optimized CS loaded NLCs was found to be  $60.45 \pm 2.32$  nm with 0.143 PDI value as shown in the Figure 32. The presence of optimum amount of (1%) surfactant i.e T-20, reduced the particle size. Furthermore, the presence of the T-HP also influenced the reduction of the particle size. The observed PDI value less than the 0.4, which indicated the optimized CS loaded formulation, having similar size of the particles throughout the dispersion system.



**Figure 32.** PS of optimized CS loaded NLC

### 5.3.3 ZP of optimized CS NLCs

The ZP of optimized CS NLCs were found to be  $-23.5 \pm 5.72$  mV, which was shown in the Figure 33. The negative ZP of formulation is an indication that the presence of the repulsive forces in between the particles and the dispersion system was more stable. According to the DLVO theory repulsive forces are more dominating than vander walls attractive forces, it forms a more stable formulation (311)



**Figure 33.** ZP of the optimized CS loaded NLCs

#### 5.3.4 Determination of % EE and %DL of CS loaded NLCs

The percentage entrapment efficiency of CS was found to be  $98 \pm 0.23$  % and drug loading was found to be  $97.41 \pm 2.56$  %

#### 5.4 Development of CS-PB NLCs

The CS-loaded PB NLCs was developed by the procedure mentioned in the section 4.2.1.8. The developed formulation was shown in the Figure 34



**Figure 34.** CS-PB-NLCs

## 5.5 Characterization of CS loaded PB NLCs

### 5.5.1 PS and PDI of CS-PB NLCs

The PS of the CS-PB NLCs was found to be  $62.94 \pm 3.62$  nm, with 0.280 PDI value which was shown in the Figure 35. It is pertinent to add that is no significant impact on the PS of CS-PB NLCs upon addition of PB NLCs. By the observation of the PS of the CS–PB NLCs it was concluded that the formulation was in nano size range.

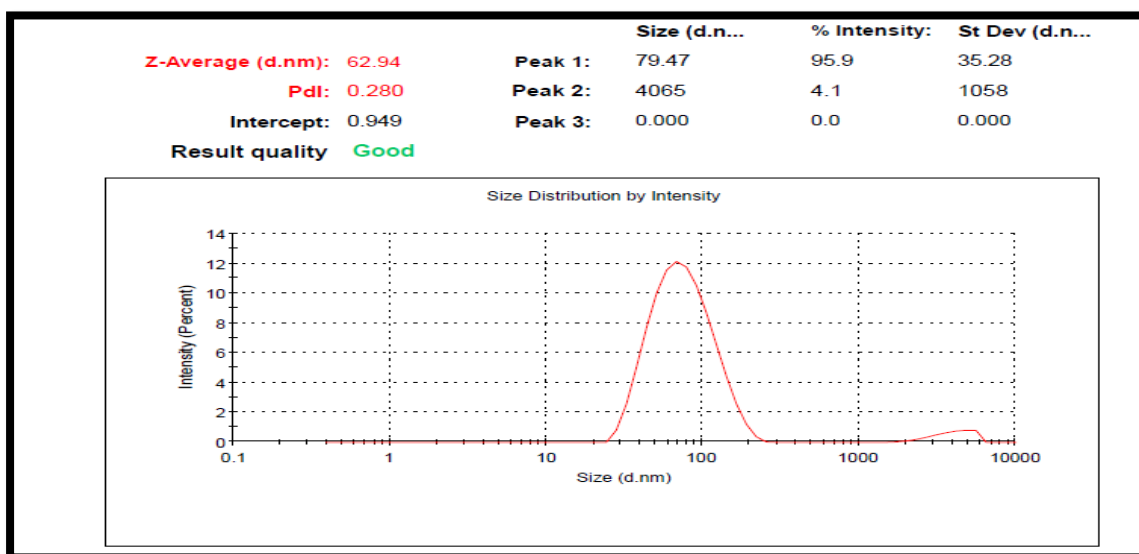


Figure 35. PS of the CS-PB NLCs

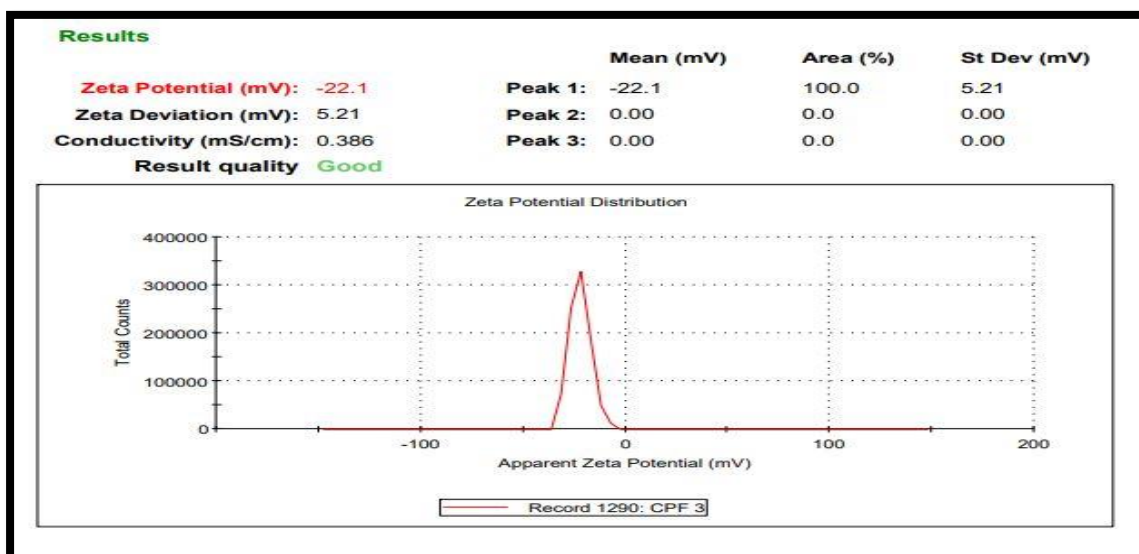


Figure 36. ZP of the CS-PB NLCs

### 5.5.2 ZP of CS-PB NLCs

The ZP of CS-PB NLCs was found to be  $-22.1 \pm 5.21$  mV, which was shown in the **Figure 36**. It is important to state here that, ideally  $\pm 30$  mV of ZP is required to stabilize a nanoparticle. However, there are many studies wherein successful development of NLCs have been reported with a ZP of  $\pm 15$  to  $\pm 20$  mV(320). This could be because of presence of lipids (GMS, C-90) as well as surfactants (T-20 and T-HP) due to generation of stearic as well as electrostatic stabilization to the formulation leading to the generation of permanent repulsive forces between the nanoparticles (321-322). The negative ZP of formulation is an indicate presence of the repulsive forces in between the particles and the dispersion system was more stable. According to the DLVO theory repulsive forces are more dominating than vander walls attractive forces, it forms a more stable formulation (316).

### 5.5.3 Determination of % EE and DL of CS- PB NLCs

% EE refers to the quantity of drug encapsulated when compared with the total amount of drug added initially. % DL defined as the quantity of drug encapsulated when compared with the amount of total lipids added in dispersion (323). The % EE of the CS-PB NLCs was found to be  $97.25 \pm 0.15$  %. This may be due to the proportion of SLs to LLs ratio as well as the type of LL used i.e C-90. In addition, lower molecular weight (CS: 254.4), higher solubility of drug in lipid might helped to obtain higher % EE. It may also attributed due to the formation of imperfections in the lipid matrix (I.T. Mendes, 2019)The % DL of the CS in CS-PB NLCs was found to be  $82.3 \pm 1.04$  %. It may be due to the formation of imperfections by the SL and LL mixture, which offers more space for loading of drug (324). This may also attributed to the higher solubility of CS in lipid matrix (325)

### 5.6 Incorporation of the CS-PB NLCs in a secondary vehicle

The prepared CS-PB NLCs were incorporated into gel by employing 0.5 % carbopol 940 the detailed information about the preparation of gel was given in the section 4.3. The developed gel was shown in the **Figure 37**



**Figure 37** CS NLCs –PB loaded gel

## **5.7 Characterization of CS NLCs- PB loaded gel**

### **5.7.1 Appearance**

The developed gel was evaluated visually, it was colour less, transparent and semi solid in nature

### **5.7.2 Spreadability**

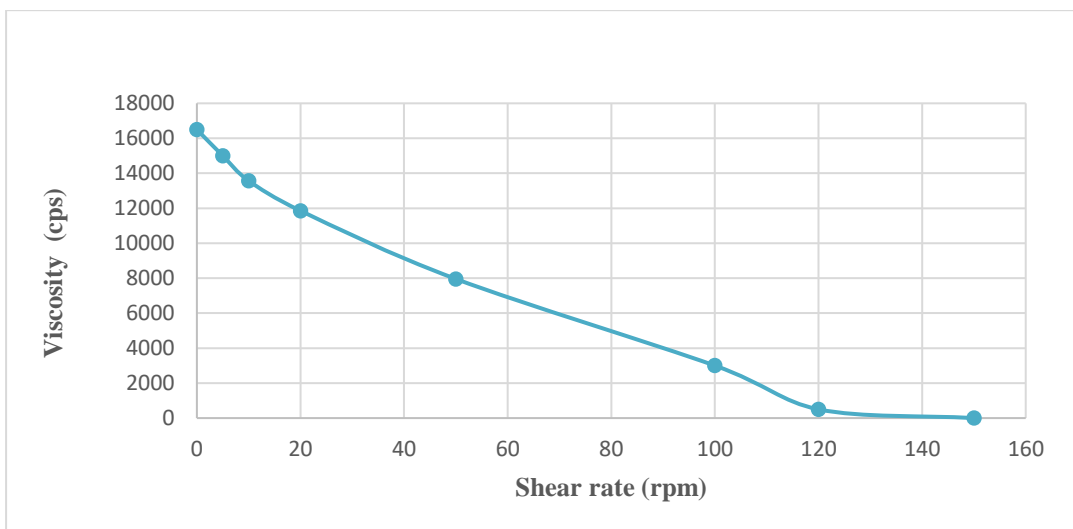
The spreading of the gel was found 4 cm in diameter which indicates that formulations have the good spreadability with proper distribution.

### **5.7.3 pH**

The pH of the developed formulation gel was measured using the digital pH meter and it was found to be  $6.8 \pm 0.36$ . It infers that CS NLCs- PB loaded gel results were nearer to that of pH of skin. It indicated that pH of formulation was compatible with pH of skin.

### **5.7.4 Viscosity**

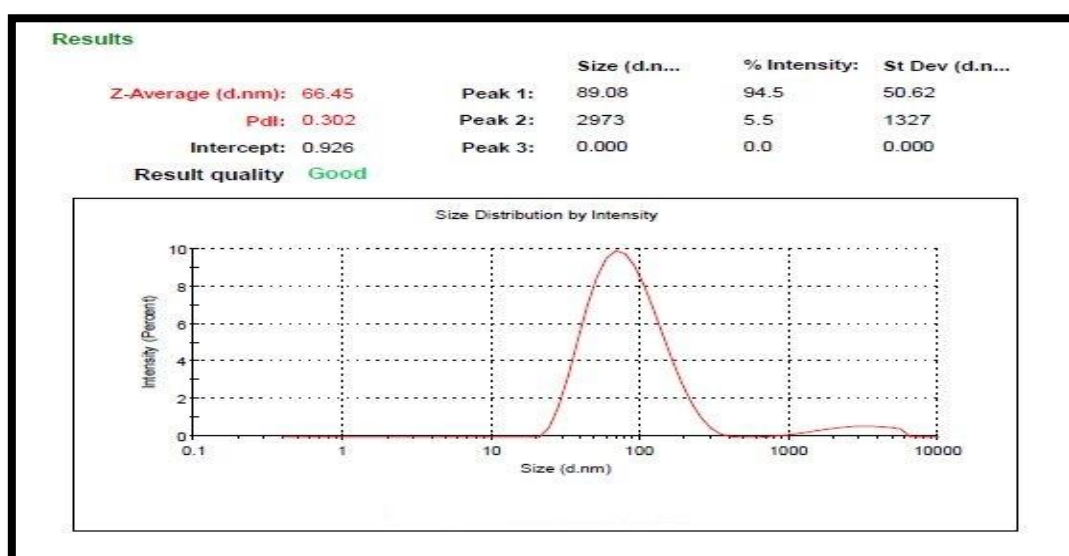
The viscosity of optimized CS-NLCs PB loaded gel was found to be in the range of 16500-500 cps. The decreased viscosity of formulation was observed as the shear rate is increased which suggesting the shear thinning property. It confirms the pseudoplastic flow behaviour from Figure 38 rheogram. It denoted that utilization of gel on the surface of skin with sufficient adherence could occur. It would increase the absorption of gel in to the skin with improved contact angle.(306)



**Figure 38** Rheogram of optimized CS-NLCs PB loaded gel

### 5.7.5 PS and PDI of CS NLCs-PB loaded gel

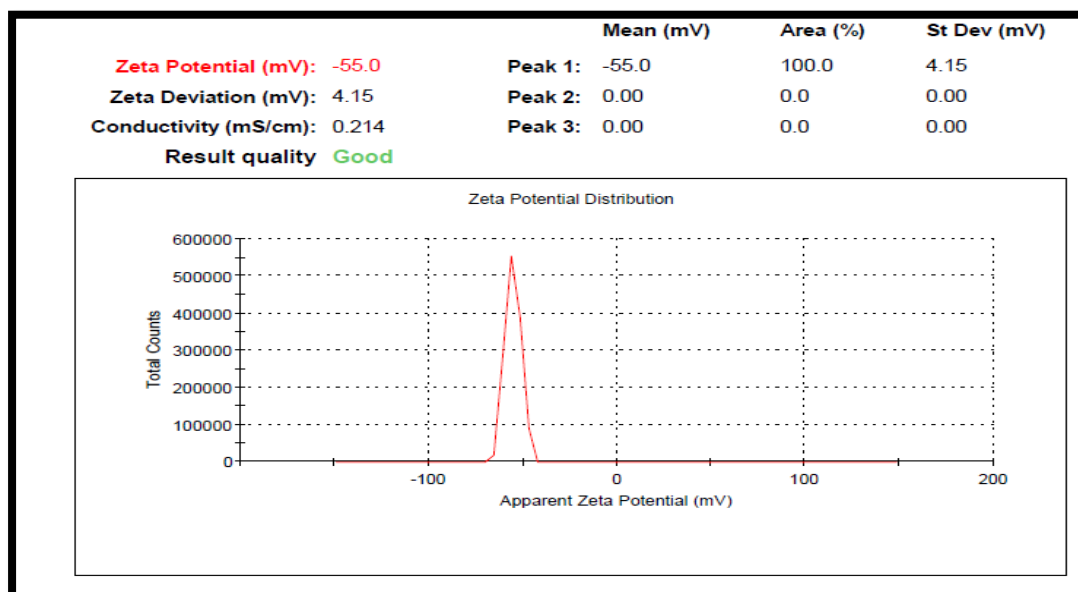
The PS of the CS NLCs-PB loaded gel was found to be  $66.45 \pm 5.62$  nm, with a PDI value of 0.302 which was shown in the Figure 39. It is pertinent to add that there is no impact on the PS of CS NLCs-PB loaded gel upon addition of carbopol 940. By the observation of the PS of the CS NLCs-PB loaded gel, it was concluded that the formulation was in nano size range.



**Figure 39** PS of the CS NLCs-PB loaded gel

### 5.7.6 ZP of CS NLCs-PB loaded gel

The ZP of **CS NLCs-PB loaded gel** was found to be  $-55.0 \pm 4.15$  mV, which was shown in the Figure 40. It is important to state here that, ideally  $\pm 30$  mV of ZP is required to stabilize a nanoparticle. In our study, the results of CS NLCs-PB loaded gel reported the ZP in the range of  $\pm 30$  to  $\pm 60$  mV, which shows the good stability of formulation (Singh *et al.*, 2011). It could be presence of lipids (GMS, C-90), surfactants (T-20 and T-HP), as well as viscosity enhancing agent i.e carbopol 940 due to which generation of stearic as well as electrostatic stabilization to the formulation leading to the generation of permanent repulsive forces between the nanoparticles (321,322). The negative ZP of formulation is an indicate presence of the repulsive forces in between the particles and the dispersion system was more stable. According to the DLVO theory repulsive forces are more dominating than vander walls attractive forces, it forms a more stable formulation (316).

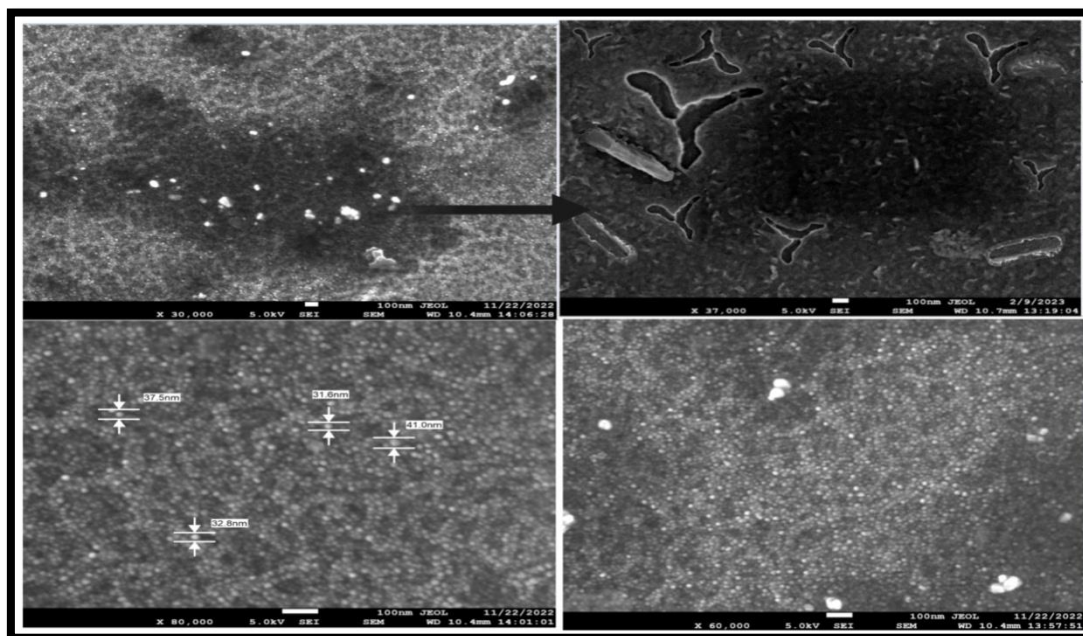


**Figure 40.** ZP of the CS NLCs-PB loaded gel

### 5.7.7 SEM study of CS NLCs-PB loaded gel

The morphological structure of **CS NLCs-PB loaded gel** was found to be smooth sphere shaped with nano size range of particles. In addition, probiotic was observed

which were appeared in the form of rod or y shaped. The SEM images of CS NLCs-PB loaded gel were shown in the Figure 41



**Figure 41.** SEM images of CS NLCs-PB loaded gel

## 5.8 Evaluation

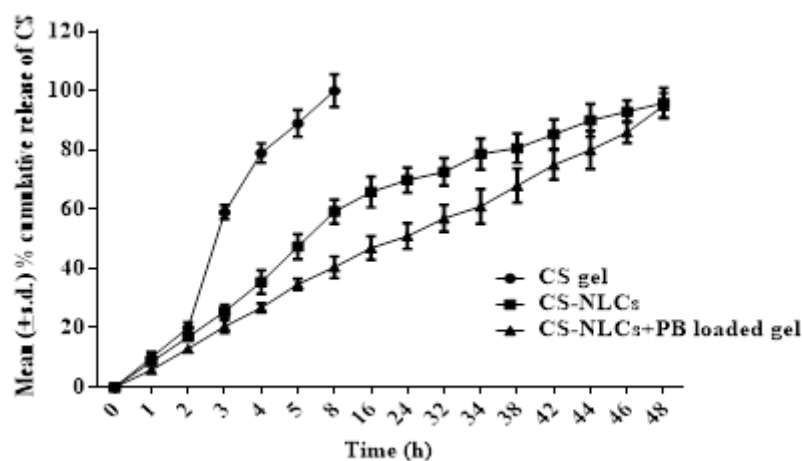
### 5.8.1 *In vitro* diffusion studies

The study was aimed to evaluate release effect of CS NLCs-PB loaded gel in order to get a prolonged release of CS at the site of skin disease like psoriasis with better spreadability of drug on the wound area. The release study was conducted for naive CS gel, CS-NLCs and CS NLCs-PB loaded gel. It was given in the **Figure 42**. It was inferred from the study that in the initial 8 h of the study, 100% of CS got released from the naive CS gel. This indicated its faster release profile, it could be due to its conventional form. However, the release of CS from CS-NLCs was about  $55.24 \pm 4.11$  % and CS from CS NLCs- PB loaded gel was about  $40.47 \pm 3.65$  % in the initial 8 h. This indicated a sustained release profile of CS NLCs and CS NLCs-PB loaded gel. The decrease in release of CS from CS NLCs and CS NLCs-PB loaded gel as compared to naive CS gel was found to be 1.81-fold and 2.5 folds respectively.



This may be due to matrix effect of GMS and CAP-90, which has impact on liquid flow and drug release as it delays free diffusion"(326)

Similar types of results were observed in previous study also (327). It was also observed that the release of CS from CS NLCs-PB loaded gel was about 1.37-fold lesser as that of CS-NLCs. This could be due to the presence of polysaccharides, that present in the PB blend that would have further restricted the release of CS from the CS NLCs-PB loaded gel. This was attributed due the intact swollen microparticles of polysaccharides which hinders the release of CS by holding it within the matrix (328)



**Figure 42.** In vitro release study of the CS

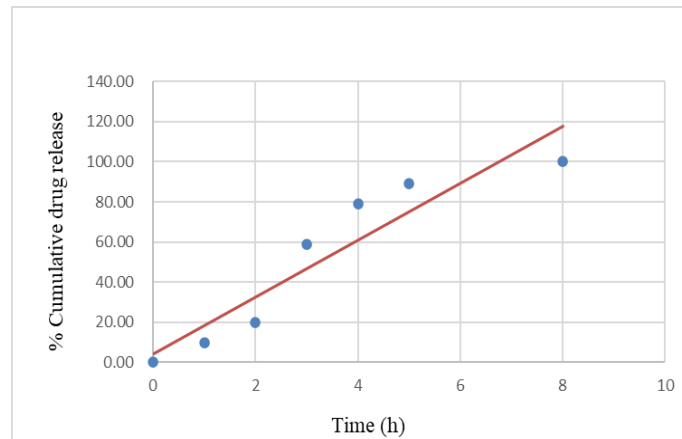
### 5.8.2 Release kinetics study

The release pattern of CS gel, CS NLCs and CS-NLCs-PB loaded gel was investigated through different release kinetic release studies i.e., “zero order”, “first order”, “Hixson Crowel”, “Higuchi’s, Weibull, Bakers Lonsdale and Korsmayer-Pepass” equations (328). The results revealed that release pattern of CS-NLCs-PB loaded gel did not follow first order ( $R^2$  adj.=-0.983), zero order ( $R^2$  adj.=0.935), Hixson crowel ( $R^2$  adj. = 0.967) and Bakers Lonsdale ( $R^2$  adj. 0.973) Kinetics. The release pattern was best fitted for Higuchi’s for CS-NLCs-PB loaded gel which indicated the sustained release and further it has followed Korsmayer -Pepass kinetics which indicated the release of CS through diffusion process( $n=0.5$ ). For further confirmation on release pattern of CS NLCs-PB loaded gel and CS-NLCs release

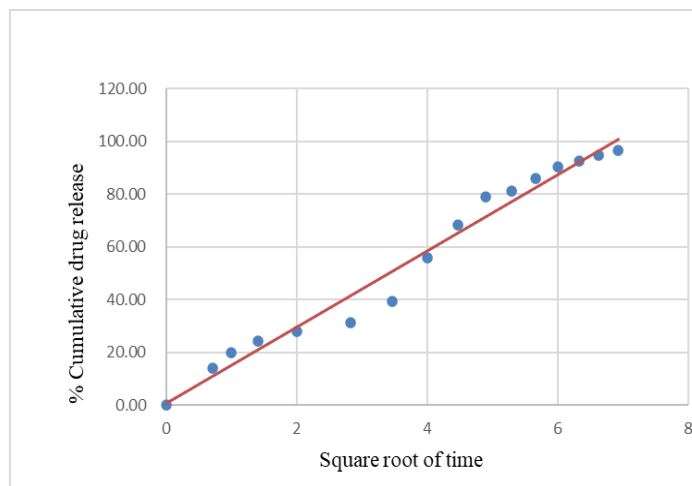
study was checked with korsmayer-pepass equation. It was found that the release exponent ‘n’ is more than 0.45 which indicated the fickian diffusion type of release from CS-NLCs-PB loaded gel(329). Moreover, several studies revealed that, “the mechanism of drug release followed drug diffusion through the lipid matrix from NLCs” (330,331). Furthermore, AIC (Akaike information criteria) and K values were confirmed the Higuchi was the best fit model for the CS NLCs PB loaded gel. The graphical representation of the kinetic models was shown in Figure 43 (zero order), 44(Higuchi model) and 45 (Higuchi model) for CS gel, CS NLCs and CS NLCs PB loaded gel respectively. The obtained release kinetics results of the CS gel, CS NLCs and CS NLCs PB gel were shown in the Table 34. As per the Weibull equation concerned no lag time was observed for the CS gel as the  $T_i$  value is negative which indicated that the CS gel has shown the instantaneous release of the CS. The CS NLCs PB loaded gel has shown the burst release initially, followed by the sustained release. In addition to that the rate of release of drug from CS NLCs and PB loaded gel was reduced up to 1.12 folds when correlated with CS gel.

**Table 34** Release kinetics of CS from CS gel, CS NLCs and CS NLCs PB loaded gel

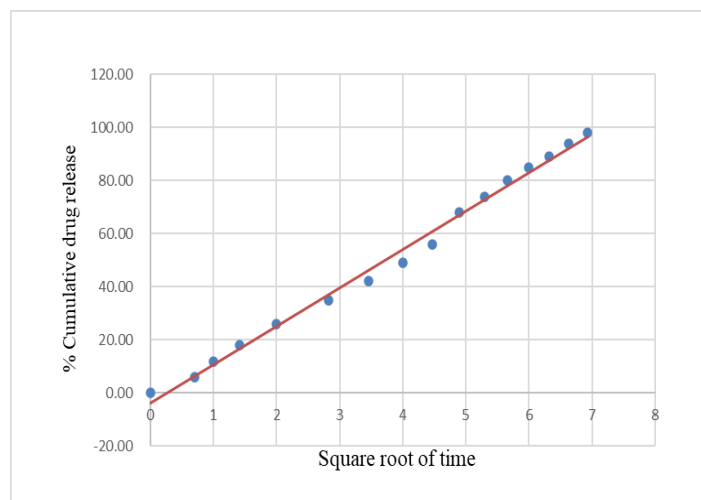
Formulation	Parameters	Zero order	First order	Higuchi	Hixson Crowell	Weibull	Bakers Lonsdale	Korsmayer pepass
CS gel	k	15.020	0.28	33.920	0.080	18.280	0.020	21.750
	R <sup>2</sup> adjusted	0.990	0.93	0.910	0.910	0.990	0.870	0.938
	AIC	52.070	51.42	53.930	48.990	37.480	56.260	52.210
	n							0.787
	$\alpha$					18.280		
	$\beta$					2.380		
	$T_i$					-0.050		
CS NLCs	k	2.220	0.04	13.080	0.010	12.310	0.006	19.197
	R <sup>2</sup> adjusted	0.780	0.94	0.910	0.900	0.980	0.980	0.976
	AIC	140.770	120.03	53.930	128.320	101.750	103.506	107.360
	n							0.399
	$\alpha$					4.320		
	$\beta$					0.570		
	$T_i$					0.860		
CS NLCs PB loaded gel	k	2.130	0.040	13.085	0.010	12.310	0.005	10.830
	R <sup>2</sup> adjusted	0.930		0.9885	0.960	0.970	0.97322	0.990
	AIC	124.06	109.340	96.930	113.270	108.40	110.35	96.320
	n							0.5000
	$\alpha$					12.31		
	$\beta$					0.820		
	$T_i$					-0.070		



**Figure 43.** zero order release kinetics of the CS gel



**Figure 44.** Higuchi square root of equation for CS NLCs



**Figure 45.** Higuchi square root of equation of CS NLCs-PB loaded gel

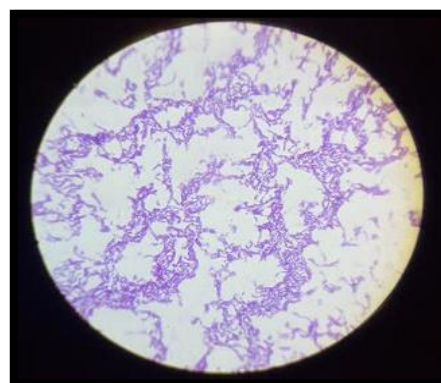
### 5.8.3 Antibacterial study

#### 5.8.3.1 Enumeration of *Staphylococcus aureus*

The colonies of *Staphylococcus aureus* were visually observed which were smooth, circular, convex, moist, 2-3 mm in diameter and gray to light coloured, shown in the Figure.46 The gram staining (Figure 47) of *Staphylococcus aureus* was appeared in purple-coloured chains and clusters. It indicated that *Staphylococcus aureus* was gram positive cocci, grew in clusters, pairs and also occasionally in chains.



**Figure 46** *Staphylococcus aureus* colonies



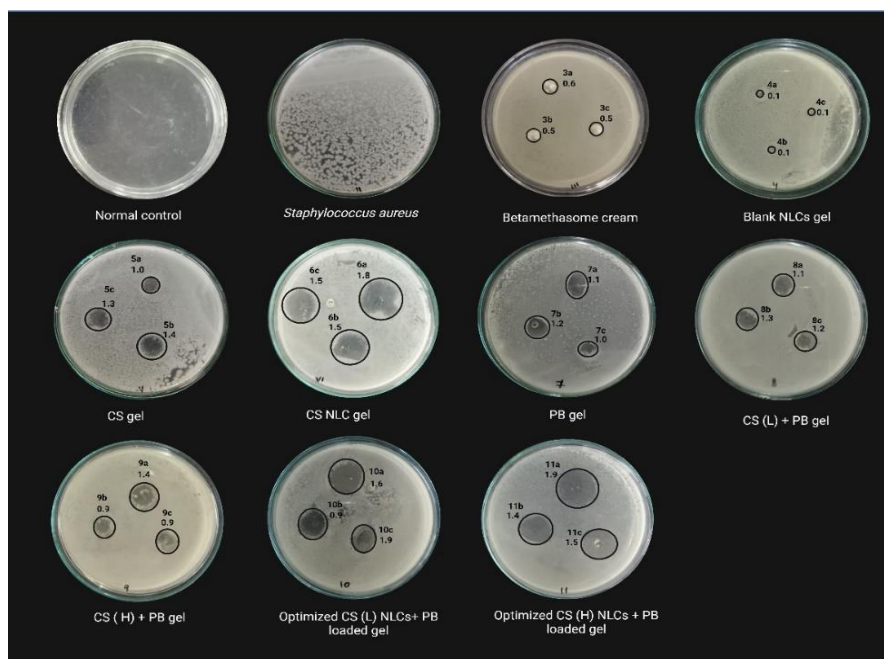
**Figure 47** Gram staining of *Staphylococcus aureus*

#### 5.8.3.2 Determination of ZOI

The antibacterial study for CS NLCs and PB loaded gel was performed by the use of *Staphylococcus aureus* (7405). The ZOI for all the treated groups was observed except for group III and IV which include marketed Betamethasone acted as positive control and blank NLCs respectively. The ZOI of CS NLCs-PB loaded gel was 0.5-fold, 0.2-fold and 0.54-fold higher than PB alone, CS alone and CS-PB combination, respectively. It indicated significantly ( $P < 0.05$ ) higher antibacterial activity of CS-PBNLCs as compared to other treatment groups, it was mentioned in the Table 35 and Figure 48

**Table 35.** Antibacterial study of the developed formulation

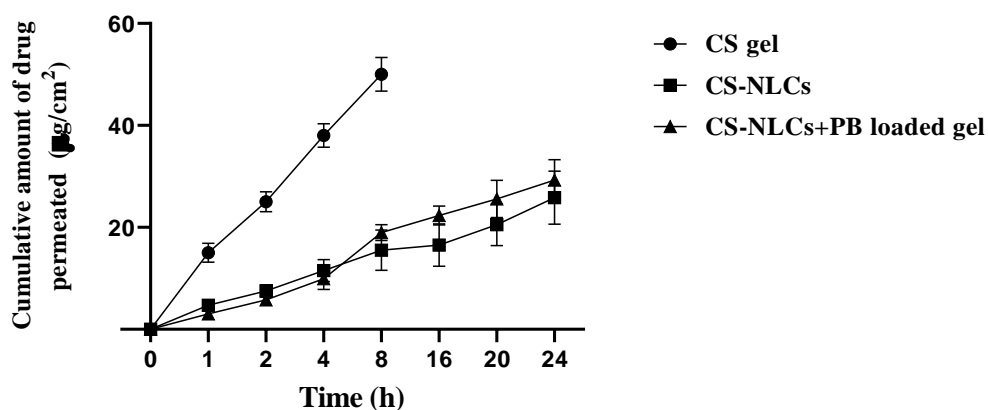
Group no.	Groups	Treatment	Dosage	No. of petriplates	Results
I	Normal control	Normal saline (no induction)	Spreaded on the solidified agar media	3	No growth was observed which indicates that medium is sterilized
<i>Staphylococcus aureus</i> spreaded on the solidified agar petriplates					
II	Experimental control	<i>Staphylococcus aureus</i> (Test organism)	50 billion CFU spreader on the solidified agar media	3	Round shaped colonies growth was observed, ( <i>Staphylococcus aureus</i> )
III	Positive control	Marketed Betamethasone cream	0.1% w/w filled in the 3 bores in each petriplate	3	No ZOI was observed
IV	Treatment I	Blank NLCs gel	Filled in the 3 bores in each petriplate	3	No ZOI was observed
V	Treatment II	CS gel	0.006% w/w of CS gel filled in the 3 bores in each petriplate	3	1.23 ± 0.20 cm diameter of ZOI was observed
VI	Treatment III	CS NLCs gel	0.006% w/w of CS NLCs gel filled in the 3 bores in each petriplate	3	1.33 ± 0.28 cm diameter of ZOI was observed
VII	Treatment IV	PB gel	10 billion CFU BI filled in the 3 bores in each petriplate	3	1.10 ± 0.10 cm diameter of ZOI was observed
VIII	Treatment V	CS (L)-PB gel	0.003% w/w of CS + 10 billion CFU BI loaded filled in the 3 bores in each petriplate	3	1.20 ± 0.10 cm diameter of ZOI was observed
IX	Treatment VI	CS (H)-PB gel	0.003% w/w of CS + 10 billions CFU BI loaded filled in the 3 bores in each petriplate	3	1.06 ± 0.28 cm
X	Treatment VII	Optimized CS NLCS (L) - PB loaded gel	0.0025% w/w of CS + 10 billions CFU BI loaded NLCs filled in the 3 bores in each petriplate	3	1.46 ± 0.51 cm diameter of ZOI was observed
XI	Treatment VIII	Optimized CS NLCs (H) - PB loaded gel	0.005% w/w of CS + 10 billion CFU BI loaded filled in the 3 bores in each petriplate	3	1.60 ± 0.26 cm diameter of ZOI was observed



**Figure 48.** ZOI for various treatment groups

#### 5.8.4 Ex vivo skin permeability study

The cumulative amount of drug permeated from CS gel, CS NLCs, CS NLCs-PB loaded gel was determined. For that the graphs were plotted by taking time on x- axis and cumulative amount of drug permeated through rat skin on y- axis. It was shown in the Figure 49. CS gel, permeability was faster than the CS NLCs, it was due to the lipophilic nature of the CS.



**Figure 49** Ex vivo study

In case of CS NLCs the permeability was delayed by the lipid core, which was surrounded the CS.

It has been observed that the drug release rate was slow in case of CS NLCs-PB loaded gel when compared with CS gel. Thus, CS NLCs PB loaded gel provided the sustained drug release over a period of 24 h. Steady state flux was determined from the slope, which was found to be  $1.06 \mu\text{g}/\text{cm}^2/\text{h}$

#### **5.8.4.1 Skin deposition study**

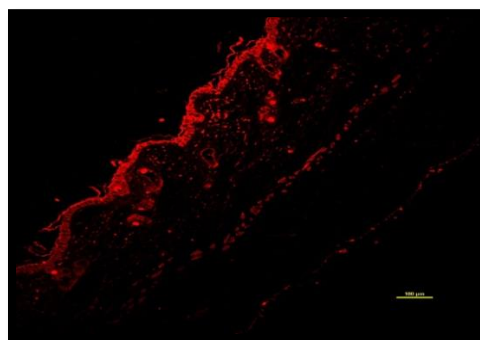
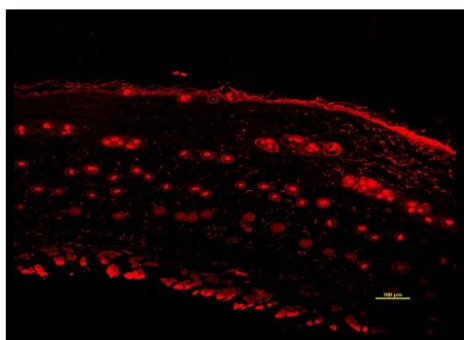
The amount of drug retained for the CS NLCs PB loaded gel is  $28 \mu\text{g}/\text{cm}^2$ . In case of CS gel the amount of CS retained on the skin is  $10 \mu\text{g}/\text{cm}^2$ . It was indicated that the higher skin deposition of CS was observed in case of CS NLCs PB loaded gel that was 2.8 folds higher than that of CS gel. It was indicated that the amount of CS was retained on the stratum corneum was higher and which also shows the sustained effect.

#### **5.8.4.2 Modified draize test**

The blank gel application on to the mice skin does not show the any irritable effect such as erythema, dryness and oedema upon repeated exposure up to 14 days which indicated that the excipients used in the preparation of the blank gel does not has any irritable effect and conforms their safety.

#### **5.8.4.3 Cellular uptake study of drug**

The cellular uptake study of the drug was determined by CLSM. The prepared RB (0.1%) and RBNLC gel were examined by CLSM, it analysed the depth of cellular uptake. The mechanism and depth of penetration involved in the RB and RB NLCs gel was demonstrated in the Figure 50 and 51



**Figure 50 CLSM in rat skin (RB)      Figure 51 CLSM in rat skin (RB NLCs gel)**

RB has shown the highest intense fluorescence in epidermis and dermis of the rat skin. In case of RB NLCs gel much greater intensity of fluorescent was observed in stratum corneum it indicated as, NLCs has higher skin deposition. However, a lowest intense fluorescence was observed in dermis as well.

## **5.9 *In vivo* study**

### **5.9.1 Evaluation parameters of anti-psoriatic activity**

#### **5.9.1.1 PASI**

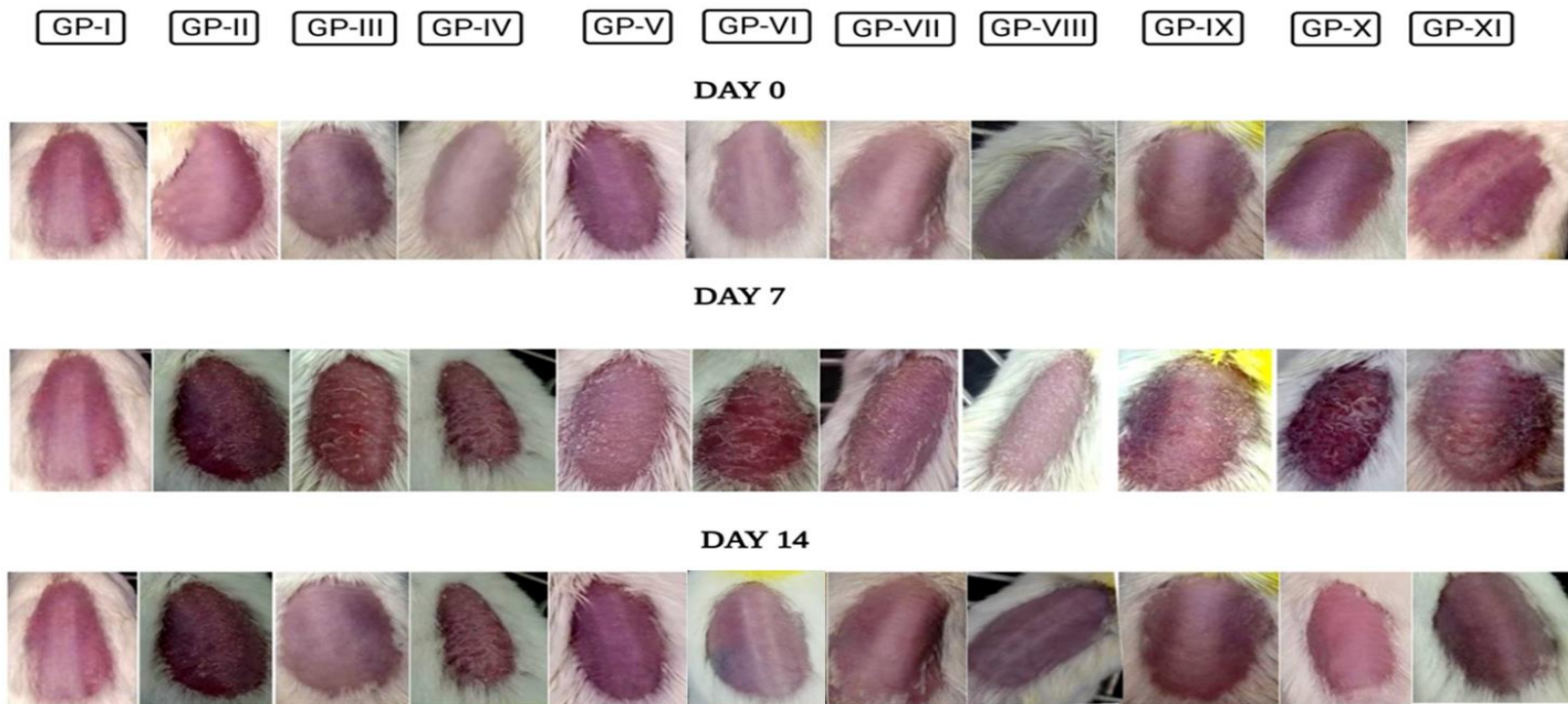
To induce the psoriasis IMQ induced cream (62.5 mg) was applied to all groups except GP-1. At the period of induction time (From 0 to 7 days) visually observed the formation of the erythema, thickness and scaling. Then the photographic images of all groups were taken from day zero to seven i.e induction time. After induction, from 8<sup>th</sup> day onwards treatment has given as mentioned in study design up to 14<sup>th</sup> day. The severity of scoring in all groups except GP-1 was scored. The erythema, of skin become a more apparent on second day onwards, however from day 3<sup>rd</sup> onwards scales are more visible in all imQ applied groups along with erythema when compared with normal group. A significant increase in erythema and scales were observed on day 4. In case of experimental control and blank NLCs gel no change in symptoms i.e erythema and scales was observed from day 2 to day 14. (332)

From day 8<sup>th</sup> to 14<sup>th</sup> the reduction of erythema, scaling and skin thick ness was recorded. The diagrammatic representation of anti-psoriatic study of day 0 to 7<sup>th</sup> day and 14<sup>th</sup> (induction and treatment) were shown in **Figure 52 and**

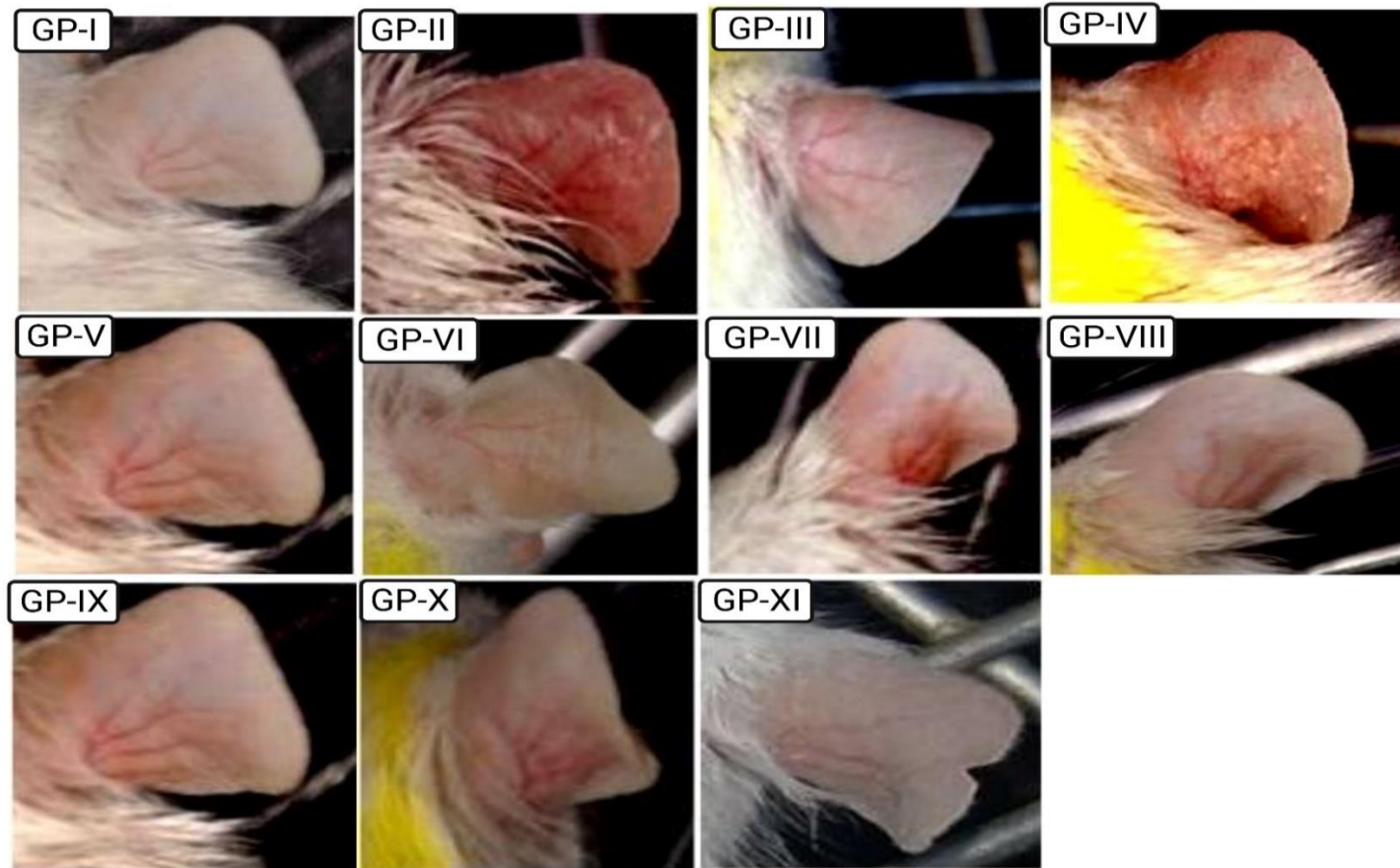


**53.** All treatment groups were compared (GP 3, 5-GP11) with normal (GP-1) as well as experimental control groups (GP-2). It has been observed visually all treatment groups reduced the psoriatic features on dorsal skin and ear when compared with experimental control (GP-2). The reduction of the scoring scale i.e erythema, scaling and thickness for all groups was represented in the graphical form (**Figure 54 a, b and c**). However, the efficacy of treatment was varied from one group to another group. The severity of PASI (Erythema, Scaling and Thickness) reduction of all treated groups was given here descending order as follows

**GPXI>GPX>GPIII>GPIX>GPVIII>GPVI>GPVII>GPV.** The optimized CS<sub>H</sub> NLCs-PB loaded gel was significantly reduced the severity of PASI and which was less from day 8<sup>th</sup> to 14<sup>th</sup> as compared with experimental control and also standard group, validating its potential in reduction of PASI. The observed results with GP-X and GP-XI were more efficacious than the standard control and significant difference was observed when compared with experimental control (p<0.001). However other groups reduced the PASI but less effective as compared with NLCs based gel. (332)

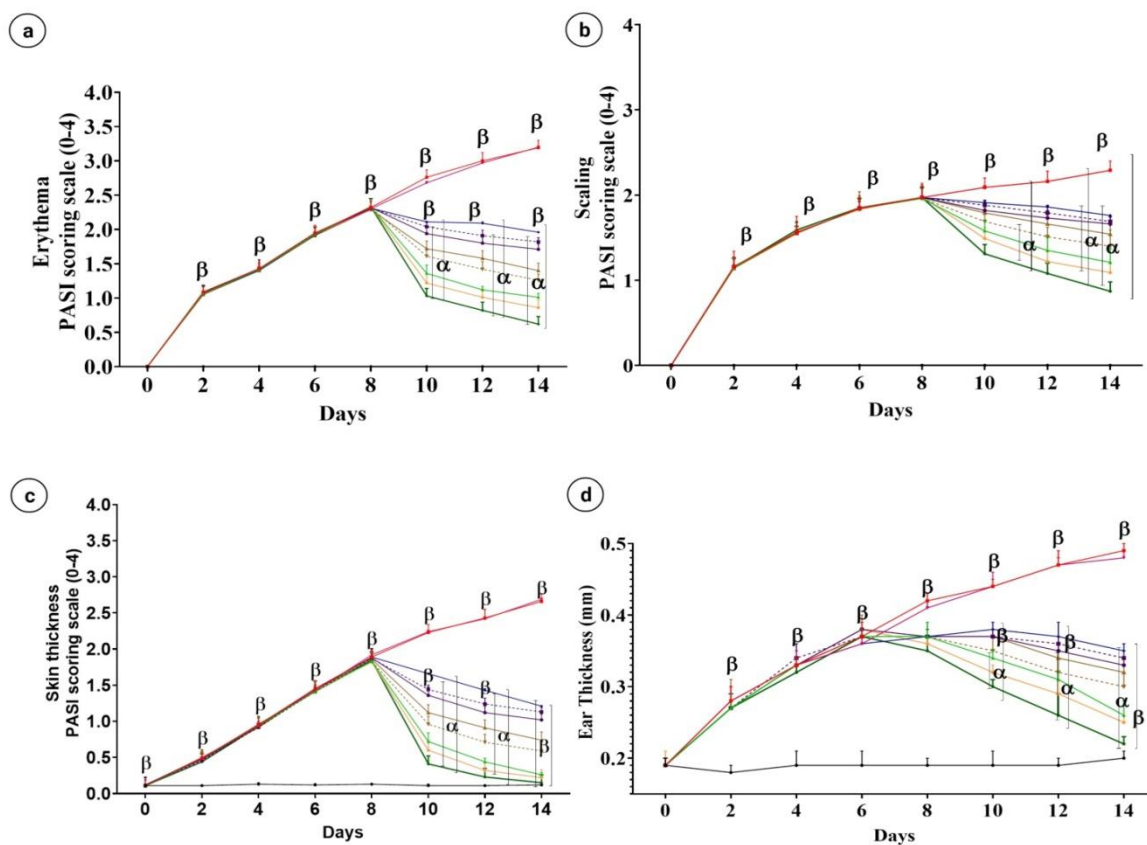


**Figure 52** Diagrammatic representation of antipsoriatic activity on mouse skin



**Figure 53** Diagrammatic representation of anti-psoriatic activity on mouse ear

- Normal Control (GP I)
- Experimental Control (GP II)
- Positive Control (GP III)
- Blank NLC gel (GP IV)
- CS gel (GP V)
- CS NLC gel (GP VI)
- PB gel (GP VII)
- CS L- PB gel (GP VIII)
- CS H-PB gel (GP IX)
- Optimized CS L NLCs-PB loaded gel (GP X)
- Optimized CS H loaded NLCs- PB gel (GP XI)



**Figure 54. Graphical representation of reduction of a. Erythema, b. Scaling c. Skin thickness d. and Ear thickness in treated groups.**

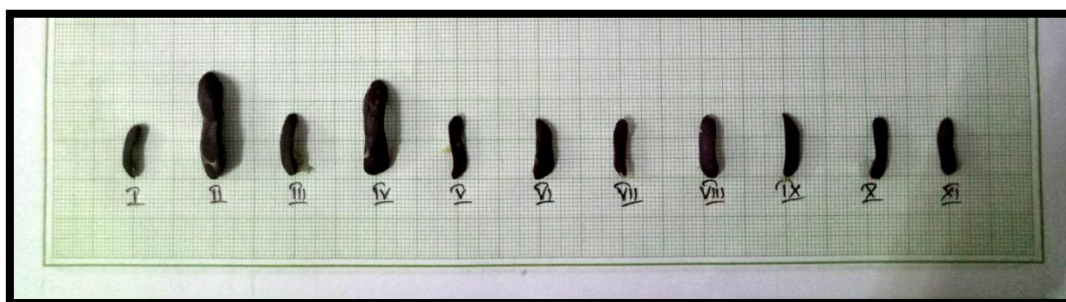
### 5.9.1.2 Ear thickness

At the induction time, the thickness of right ear increased significantly in all ImQ induced groups. The reduction of ear thickness of all groups (GP3, 5-GP11) at the treatment period was recorded. In case of NLCs containing CS and PB treated group the significant reduction (GP-10 and 11) of the ear thickness was observed. However other treated groups also shown the effect in the reduction of ear thickness but not completely normalized as compared with NLCs containing formulations. In case of NLCs the least difference was observed which was nearer to normal group value indicated the insignificant in ear thickness and significant difference with that of experimental group ( $p>0.001$ ). It was promising that NLCs based formulation regained the normal thickness of ear when compared with other formulations. The order of the reduction of the ear thickness at the treatment period (From 8<sup>th</sup> -14<sup>th</sup> day) was given in the following descending order **GPXI>GPX>GPIII>GPIX>GPVIII>GPVI>GPVII>GPV**. The results were shown, graphically by taking days on x-axis and ear thickness on y-axis (Figure 54.d) (333-335)

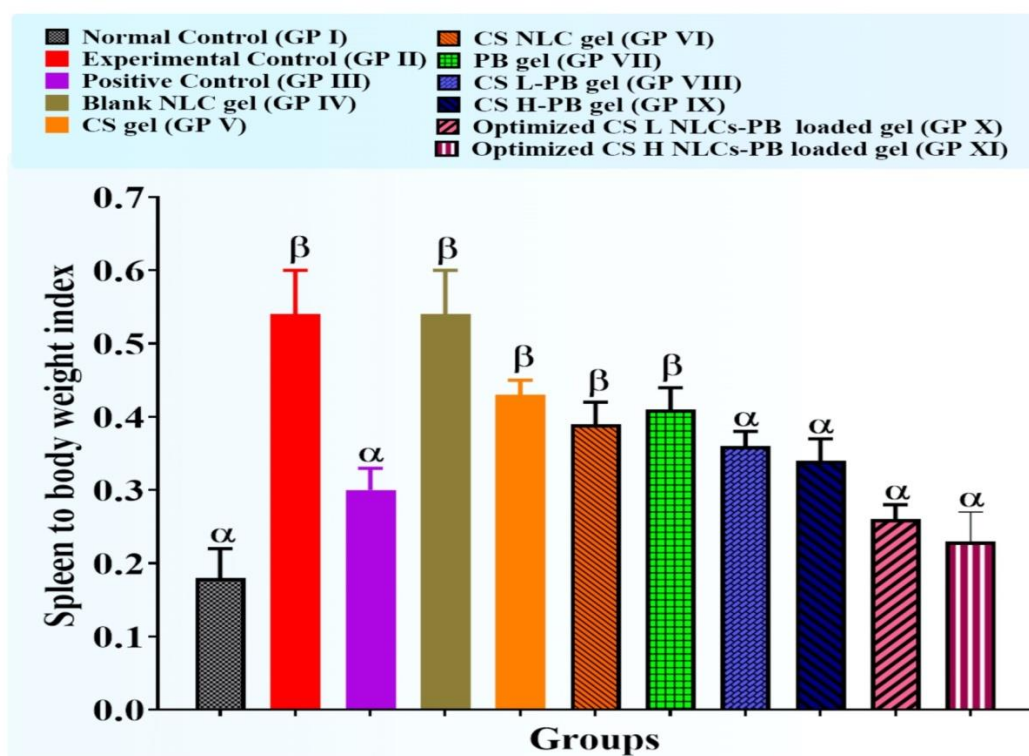
### 5.9.1.3 Spleen to body weight index

After the completion of treatment, all groups' animals were sacrificed with cervical dislocation and then spleen was collected and washed with saline. Then photographic spleen images of all groups were taken, it was shown in the **Figure 55** Later, immediately spleen weight of all groups was recorded. The weight and size of the spleen (Spleno-megaly) was significantly increased in experimental control (GP-2). It could be due to the increased levels of the cytokines (IL-17/23) (333-335). In case of the treatment groups spleen to body weight ratio was reduced significantly when compared with experimental group (GP-2). However, the ratio of the reduction of spleno-megaly was depends upon the type of formulation used for treatment. In case of the NLCs containing groups the reduction of spleno-megaly was non-significant with that of normal control (GP-1) and significant with that of the experimental control (GP-2). It was observed that the amelioration of cytokines in the spleen was based on the dose as well as type of formulation used in the treatment.

The significant reduction of splenomegaly was observed in ascending order **GP-XI** > **GP-X** > **GP-III** > **GP-IX** > **GP-VIII** > **GP-VI** > **GP-VII** > **GP-V** which was shown in the **Figure 56** The significant difference was observed between the experimental control and treated groups ( $p > 0.001$ ).



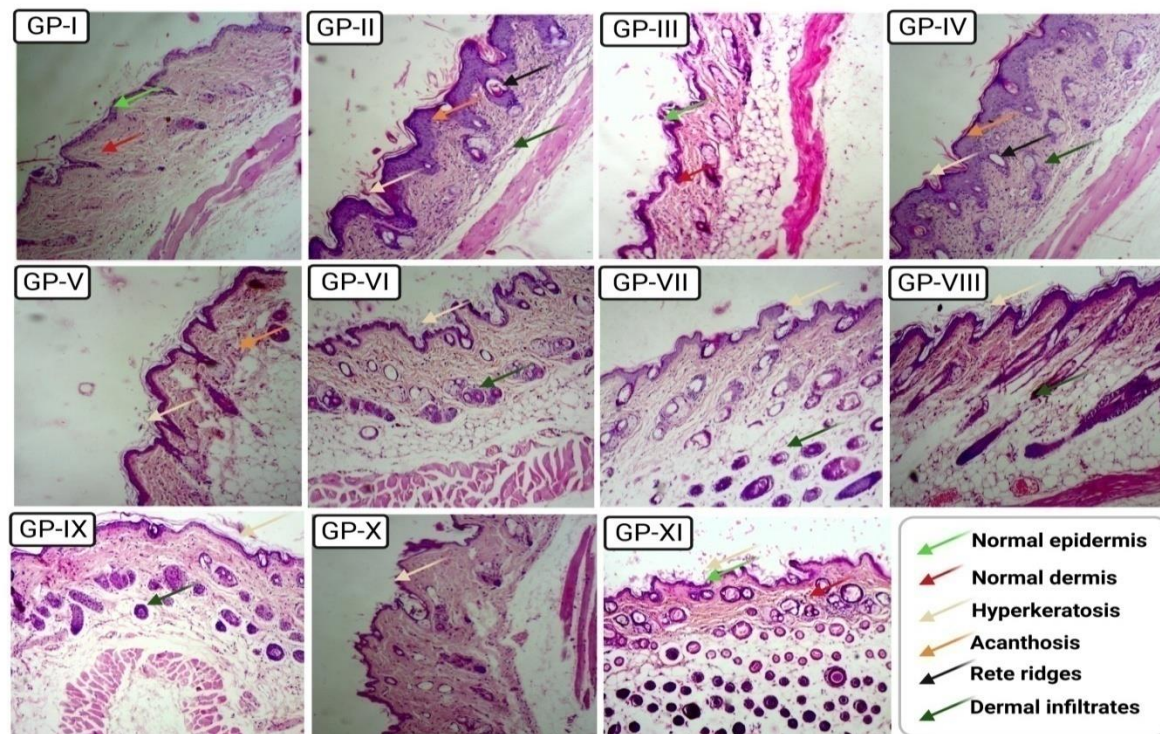
**Figure 55.** Images of spleen



**Figure 56** Spleen to body weight

#### 5.9.1.4 Histopathological study

The histology of the ear and skin was observed after completion of 14<sup>th</sup> day study. By analysing the transverse sections of skin and ear it has been observed that the phenotype variations were observed. The intensity of psoriasis like inflammation, acanthosis and dermal infiltration was more prominent in group 2 and also group 4. In the treatment groups decreased and absence of psoriasis features was observed (GP3,5-GP11) when compared with experimental control (GP2) ( $p > 0.001$ ). However, the efficacy of the reduction of the psoriasis features were varied from one group to another group. All the details of histological study were given in the Table 36. The histological images of dorsal skin and ear were shown in Figure 57 and 58 respectively. The overall histological study of treated group with gel containing optimized CS high dose NLCs-PB (GP-11), markedly reduced dermal infiltrates and thickness of epidermis. Furthermore, the healing of keratinocytes was better than the marketed formulation (GP-3) (Betamethasone cream).

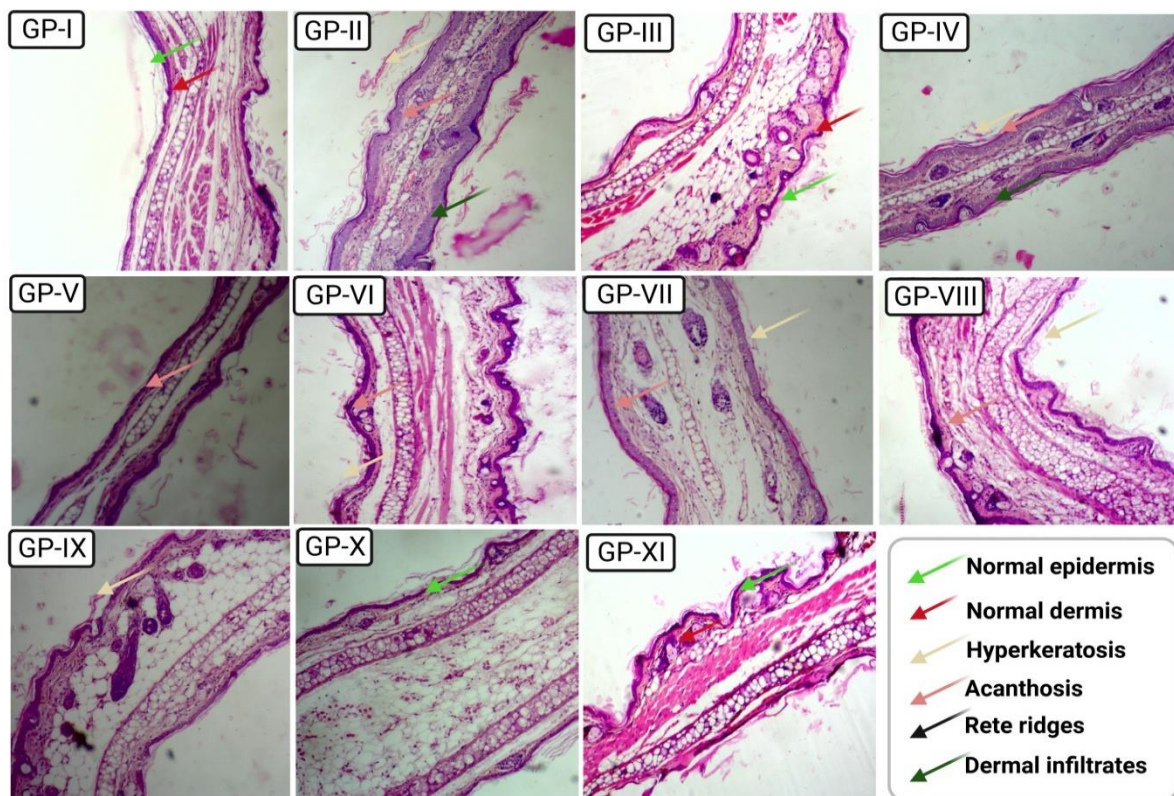


**Figure 57.** Histological study of dorsal skin of all treated groups

**Table 36.** Histological study of all treated groups

Group no.	Features of dorsal skin	Features of ear
I	Normal Epidermis Normal Dermis	Normal Epidermis Normal dermis
II	Hyperkeratosis Acanthosis Higher epidermal and dermal infiltration More prominent rete ridges	Hyperkeratosis Acanthosis Higher epidermal and dermal infiltration and rete ridges
III	No acanthosis No epidermis and dermal infiltrations	No acanthosis No epidermis and dermal infiltration
IV	Hyperkeratosis Acanthosis Higher epidermal and dermal infiltration More prominent blood vessels and rete ridges	Hyperkeratosis Acanthosis Higher epidermal and dermal infiltration More prominent blood vessels and rete ridges
V	Mild reduction of acanthosis, moderated reduction of epidermal and dermal infiltrations	Mild reduction of acanthosis moderated reduction of epidermal and dermal infiltrations
VI	Moderate reduction of acanthosis, moderated reduction of epidermal and dermal infiltrations when compared with normal control as well as standard group	Moderate reduction of acanthosis, moderated reduction of epidermal and dermal infiltrations when compared with normal control as well as standard group
VII	Moderate reduction of acanthosis, moderated reduction of epidermal and dermal infiltrations when compared with normal control as well as standard group	Moderate reduction of acanthosis, moderated reduction of epidermal and dermal infiltrations when compared with normal control as well as standard group
VIII	Mild reduction of acanthosis, mild reduction of epidermal and dermal infiltrations	Mild reduction of acanthosis, mild reduction of epidermal and dermal infiltrations
IX	Mild reduction of acanthosis, moderate reduction of epidermal and dermal infiltrations	Mild reduction of acanthosis, moderate reduction of epidermal and dermal infiltrations
X	Moderate reduction of acanthosis, moderate reduction of epidermal and dermal infiltrations	Moderate reduction of acanthosis, moderate reduction of epidermal and dermal infiltrations
XI	Complete reduction of acanthosis, epidermal and dermal infiltrations. It showed the similar type of features with that of normal and standard group. It indicated dose dependant antipsoriatic activity	Complete reduction of acanthosis, epidermal and dermal infiltrations. It showed the similar type of features with that of normal and standard group. It indicated that dose dependant anti- psoriatic activity





**Figure 58.** Histology of ear of all treated group

#### 5.9.1.5 Estimation of antioxidant property of developed formulation in skin

The extent of the oxidative stress in the skin samples was measured. In the experimental control group, the significant reduction of amount of catalase, SOD, GSH enzyme and increased amount of MDA was observed when compared with normal control (GP-1), which is an indication that the imQ caused the oxidative stress. The topical application of gel on the 6.25 cm<sup>2</sup> area of skin, causes the significant increment of SOD, GSH, catalase and amelioration of MDA levels was observed. However, the level of increment of SOD, GSH, catalase and reduction of MDA level was varying significantly from one group to other treated groups.

#### **5.9.1.5.1 Catalase activity**

It has been observed that, order of level of increment of catalase in all treated groups was followed in descending manner GP-XI>GP-X>GP-III>GP-IX>GP-VIII>GP-VI>GP-VII> GP-V. It was shown in **Figure 59.a**. It was significantly difference with that of experimental control ( $p>0.001$ ). The optimized CS H NLCs-PB loaded gel improve the antioxidant catalase enzyme level, when compared with marketed product (GP-3) and it was nearly equivalent with that of the normal control (GP-1). The result imply that the developed novel formulation has the potential effect in the maintainance of the imbalance of oxidative stress (333-335)

#### **5.9.1.5.2 SOD level**

In all the groups the level of SOD was measured. It has been observed in experimental control (GP-2) the level of the SOD was less when compared with normal group (GP-1). It could be due to the oxidative stress generated by the ImQ which ultimately leads to the hyper proliferation of the keratinocyte. Our finding revealed an increment in SOD levels in all treated groups (GP-3,5-GP11). However, the level of restoration of SOD was significantly different from one group to other. The level of restoration of SOD in treated groups was followed in the descending order **GP-XI>GP-X>GP-III>GP-IX>GP-VIII>GP-VI>GP-VII >GP-V** as shown in the **Figure 59.b**. In case of optimized CS H NLCs- PB loaded gel the level of SOD was significantly higher, followed by optimized CS L NLCs-PB loaded gel when compared with the marketed drug (GP-3) and also experimental group (GP-2). The overall study confirmed that the formulation was effective in the amelioration of psoriatic inflammation (333-335)

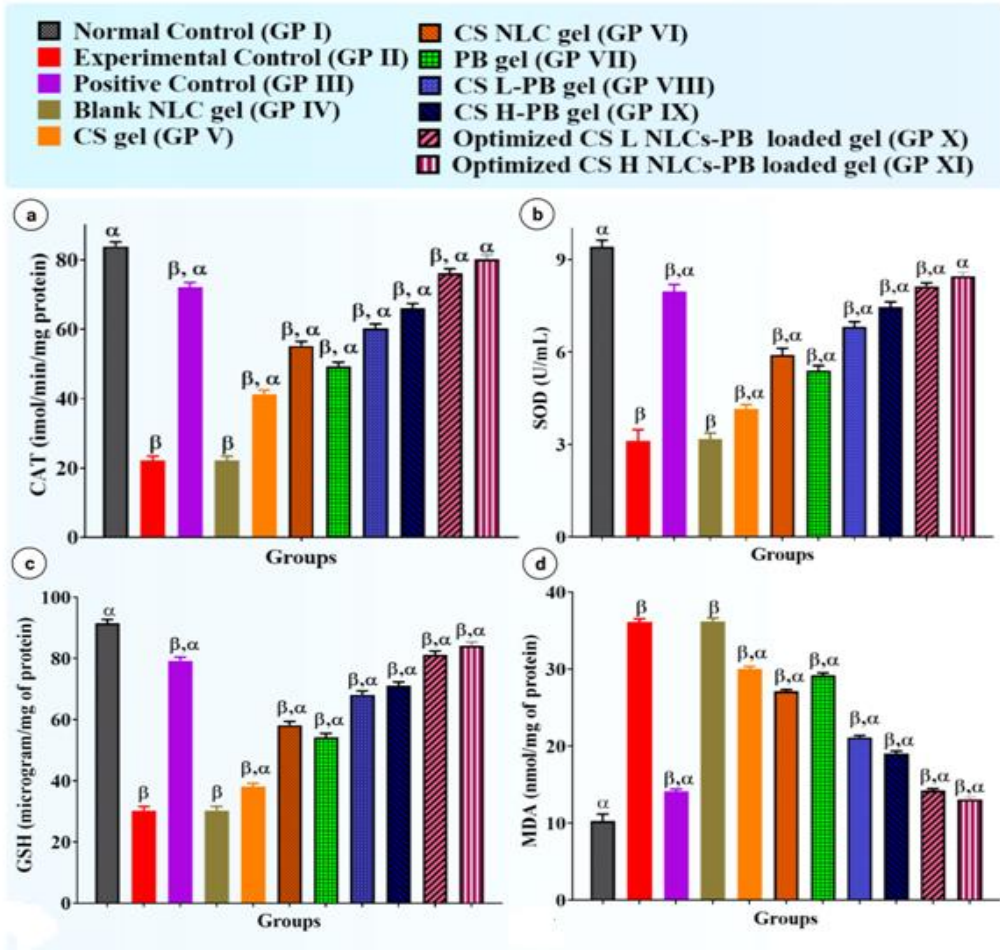
#### **5.9.1.5.3 GSH measurement**

In the experimental control group, the significant reduced level of GSH enzyme was observed when compared with normal control, which is an indication that the ImQ caused the oxidative stress. The topical application of the developed formulations on the 6.25 cm<sup>2</sup> area of skin, causes the significant increment of GSH, levels was observed. However the level of increment of GSH was varies significantly from one group to other treated groups. Briefly it has been observed that the, optimized

CS H NLCs-PB loaded gel, followed by optimized CS L NLCs-PB loaded gel, improve the antioxidant enzyme levels significantly, when compared with marketed product (GP-3) and it was equivalent to the normal control (GP-1). The Blank NLCs gel was insignificant with that of experimental control. It confirmed that blank NLCs gel was inert and also nontoxic in nature. The effects of the treatments on GSH level were shown in Figure.59. c. In case of CS gel (GP-5), PB gel (GP-7), CS L-PB gel (GP-8), CS H-PB gel(GP-9) the level of GSH was significantly less with respect with normal control. It infers the, to restore the normal level of GSH, long term treatment was required for effective treatment of the psoriasis (333-335).

#### **5.9.1.5.4 TBARS (MDA) estimation**

In the experimental control group the significant increment of level of MDA enzyme was observed when compared with normal control, which is an indication that the imQ caused the oxidative stress. It ultimately leads to the stimulation of the various inflammatory pathways such as MAPK and NF-kB. It caused the hyper proliferation of keratinocytes. The topical application of all developed formulation on the 6.25 cm<sup>2</sup> area of skin, causes the significant amelioration of MDA levels was observed. However the level of reduction of MDA level was varies significantly from one treatment group to other. The order of reduction of MDA level in the all-treatment groups was followed in the ascending order **GP-XI>GP-X>GP-III>GP-IX>GP-VIII>GP-VI>GP-VII>GP-V**. It was represented in the **Figure 59.d**. It has been observed that the optimized CS H NLCs-PB loaded gel reduced the more MDA level when compared with marketed product (GP-3) and it was nearly equivalent to the normal control (GP-1). It was significantly difference from experimental group (GP-2) (333-335)



**Figure 59.** Graphical representation of **a.** catalase; **b.** SOD; **c.** GSH; **d.** MDA levels in various treated groups.

### 5.9.1.6 ELISA assay

#### 5.9.1.6.1 Estimation of cytokines levels in the dorsal skin

##### 5.9.1.6.1.1 TNF- $\alpha$ estimation

The TNF- $\alpha$ , levels in all groups were estimated by using the ELISA kit. A significant increment in the levels of TNF- $\alpha$  was identified in experimental control (GP-2). It could be presence of higher inflammation in mouse psoriatic skin. It was indicated that the levels of significance difference were higher with normal group. The TNF- $\alpha$  levels in the treated groups were decreased with respect to that of formulation types. However, the reduction of TNF- $\alpha$  was depends upon the efficacy of the formulation used. It was shown in the **Figure 60**, the order of decreasing level of all the treated groups given in following descending order **GP-XI>GP-X>GP-III>GP-IX>GP-VIII>GP-VI>GP-VII>GP-V**. The higher significant reduction of TNF- $\alpha$  ( $p>0.001$ ) was observed in case of the optimized CS H NLC-PB loaded gel as compared with experimental group (GP-2) and equivalent to that of normal group (GP-1). It was more effective than the standard group (GP-3).

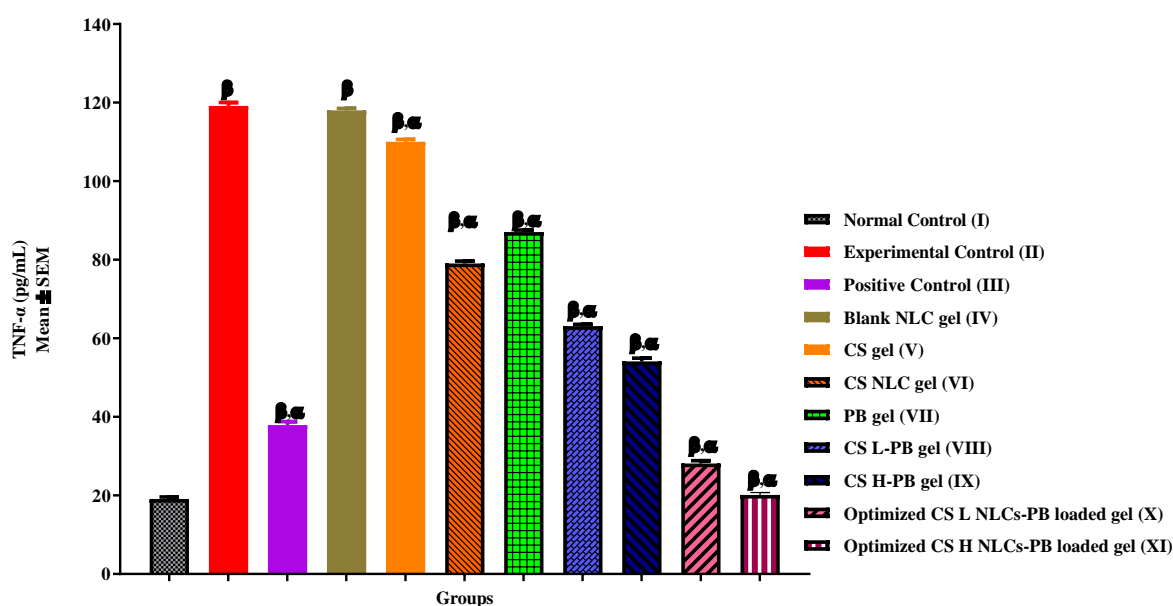
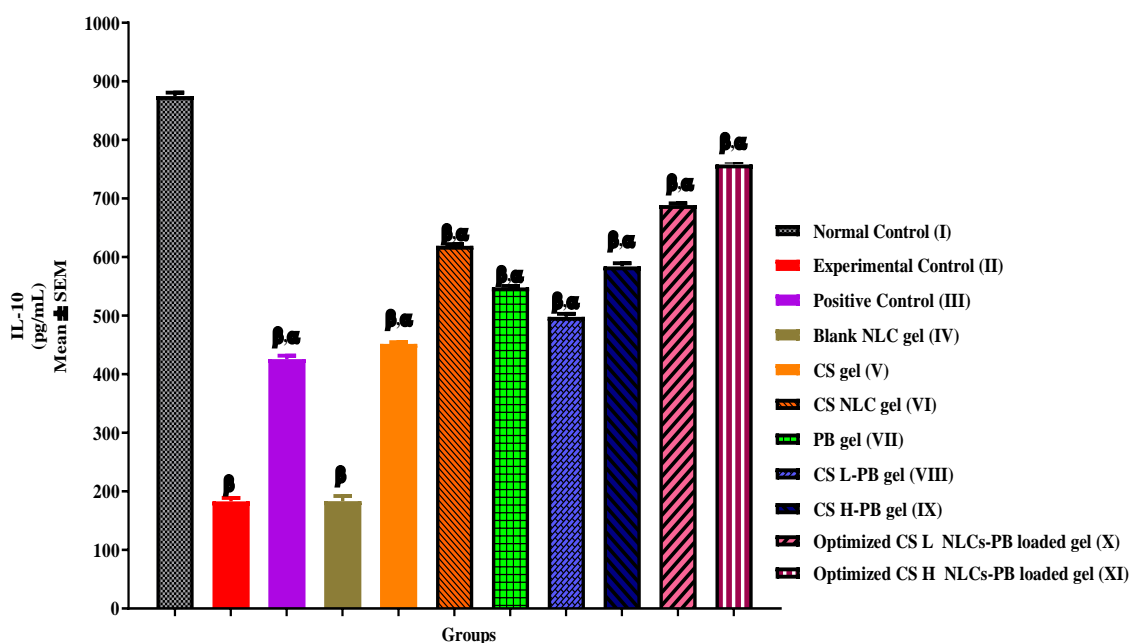


Figure 60. TNF- $\alpha$  estimation

The results were shown in Mean + SEM (Post-test- Bonferroni post hoc test). Here <sup>a</sup>p< 0.001 as compared with the Experimental control and <sup>b</sup>p<0.001 as compared with Normal control.

### 5.9.1.6.1.2 IL-10 levels

The levels of the IL-10, in all the groups were determined by using the ELISA kit. A significant reduction of IL-10 was observed in case of experimental control (GP-2) due to the inflammation caused by IMQ in mouse. The increased level of IL-10 was observed in all treated groups. However, it was identified that the level of increment of cytokine was varies from one group to other group, based on dose as well as formulation type. The levels of increment of the IL-10 were given in the following descending order **GP-XI>GP-X>GP-III>GP-IX>GP-VIII>GP-VI>GP-VII>GP-V**. However, the significant increment of IL-10 was observed in case of the optimized CS H NLC-PB loaded gel as compared with experimental group (GP-2) and equivalent to the normal group (GP-1) (p>0.001). It was shown in the **Figure 61**



**Figure 61.**IL-10 estimation

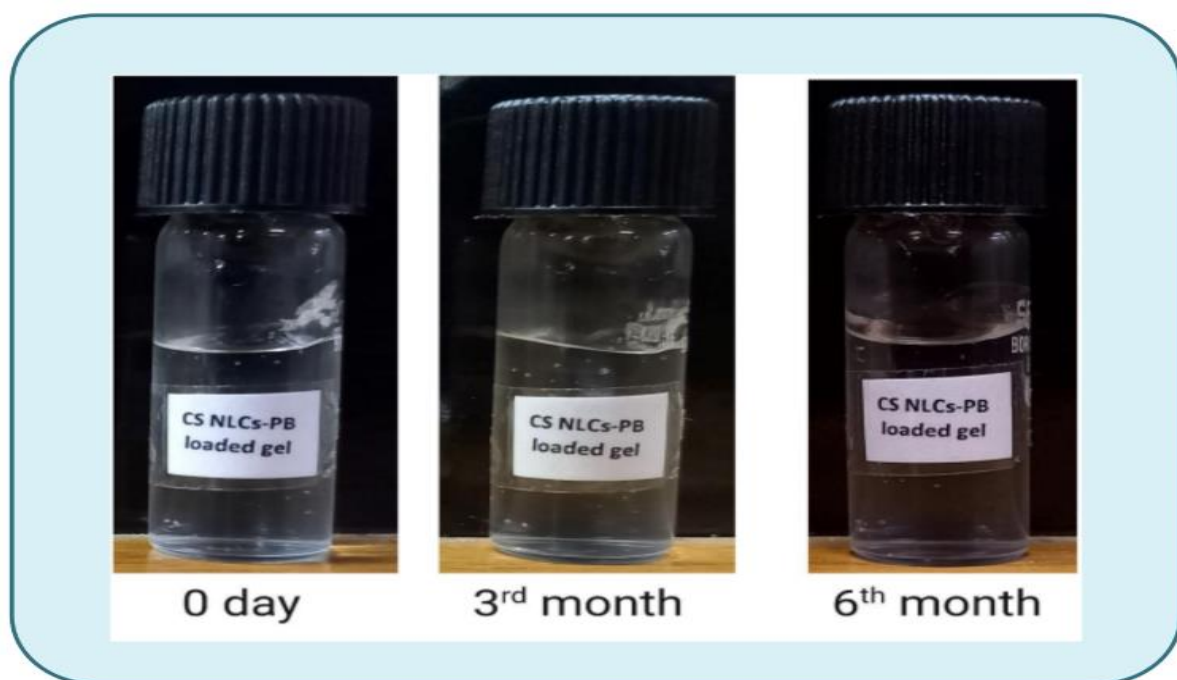
The results were shown in Mean  $\pm$  SEM (Post-test- Bonferroni post hoc test). Here  $^{\alpha}p < 0.001$  as compared with the Experimental control and  $^{\beta}p < 0.001$  as compared with Normal control.

### 5.10 Stability studies

The stability of CS NLCs –PB loaded gel was evaluated for the third and sixth month after the preparation which was shown in Table 37. No significant difference has been observed in formulation interms of physical appearance (Figure 62), PS, ZP, % EE, drug content and pH after 3<sup>rd</sup> and 6<sup>th</sup> months when compared with 0<sup>th</sup> day. It infers the developed formulation is more stable.

**Table 37.** Stability studies

<b>Formulation evaluation Time period</b>	<b>Physical appearance</b>	<b>PS nm</b>	<b>ZP mV</b>	<b>% EE</b>	<b>Drug content %</b>	<b>pH</b>
At the time of preparation	Shiny transparent	66.45 $\pm$ 5.62	-55.0 $\pm$ 4.15	98 $\pm$ 0.26	98 $\pm$ 0.11	6.8 $\pm$ 0.23
3 <sup>rd</sup> month	Shiny transparent	66.81 $\pm$ 9.23	-43.2 $\pm$ 4.13	98 $\pm$ 0.32	98 $\pm$ 0.23	6.8 $\pm$ 0.16
6 <sup>th</sup> month	Shiny transparent	73.75 $\pm$ 5.07	-42.9 $\pm$ 5.34	96 $\pm$ 0.32	97 $\pm$ 0.31	6.8 $\pm$ 0.13



**Figure 62.** Stability study of CS-NLCs PB loaded gel



# CHAPTER 6



## Summary & Conclusion

The present research work was hypothesised to develop and evaluate the CS-NLCs PB loaded gel for topical application in the management of the psoriasis. For that modified hot homogenization method and then ultra and probe sonication technique was used. Furthermore, it was converted into the gel by the addition of the carbopol 940 as a gelling agent. In this research, initially we have successfully developed and validated an analytical method by RP HPLC. The  $R_t$  of CS was found at 10.29 min. The CS RP HPLC method in between the 2-10  $\mu\text{g/mL}$  concentration was linear with value of 0.998 regression coefficient ( $R^2$ ). The developed method was accurate as it was confirmed from the mean percentage recovery was between the 95-105% at three levels. Moreover, the % RSD was found to be less than 2% confirming that the developed method was precise. "LOD" and "LOQ" were found to be 0.071 and 0.217  $\mu\text{g/mL}$ . Moreover, the validated method was robust with no significant changes in response to variation in flow rate, mobile phase composition, wavelength, and different lots of columns. The developed method was validated in terms of accuracy, precision, system suitability, and robustness according to ICH Q2 (R1) guidelines. To formulate optimized CS loaded NLCs, the parameters were selected by applying BBD. The 3D response surface plots, polynomial equations and perturbation plots helped in prediction and validation of the optimized values of selected independent variables for the formulation of CS- NLCs with desired PS, %EE and ZP. The optimized formulation was characterized for PS, ZP, %EE and %DL. The PS, ZP, % EE and % DL of optimized CS- NLCs were found to be  $60.51 \pm 5.62$  nm,  $-23 \pm 5.21$  mV,  $97.25 \pm 0.15$  and  $82.3 \pm 0.104$ , respectively. The use of optimized GMS as SL and C-90 as LL was suitable for developing optimized CS-NLCs with desired entrapment efficiency. In addition to that PS of CSNLCs were within the nano meter range. It could be due to the addition of the required amount of the surfactant and co-surfactant. Morphological study by SEM revealed that the NLCs loaded with CS were in spherical shape. Furthermore, the optimized CS-NLCs were converted in to the CS-PB NLCs, by the addition of PB in it, then analysed for the PS, ZP, % EE and % DL. The developed formulation has the, PS with 66.45nm, ZP with -22.1 mV and % EE with 97.25%. TEM study revealed that CS-PB NLCs were spherical in shape. Furthermore, it was converted in to the gel by the addition of the gelling agent i.e carbopol 940. Then it was evaluated for morphology, pH, viscosity, spreadability.

The morphological SEM study of CS-NLCs PB loaded gel was spherical in shape along with Y-shaped probiotic. The pH of the developed formulation gel was found to be  $6.8 \pm 0.36$ , which was nearer to that of pH of skin. It was concluded that the pH of formulation was compatible. The viscosity of developed CS loaded NLCs PB gel was found to be 13000 cp. It indicated that gel had adhesiveness with better cohesive property upon topical application. It could increase the absorption of gel on to the skin with improved contact angle. The *in vitro* diffusion studies revealed that  $98 \pm 0.06$  % of CS got released from CS NLCs -PB loaded gel at the end of 48 h. For initial 8h, release of CS was about 6-fold reduced in case of CS NLCs-PB loaded gel than that of naive CS gel and then the delayed release was continued up to 48h, which indicated the sustained release of CS from NLCs. The zone of inhibition of CS NLCs-PB loaded gel was 1.45-fold, 1.30-fold and 1.50-fold higher than PB gel alone, CS gel alone and CS-PB gel combination, respectively. It indicated significantly higher antibacterial activity of CS NLCs-PB loaded gel as that of any other treatment group.

The *ex vivo* permeability study of the CS NLCs PB loaded gel has shown the  $28 \mu\text{g}/\text{cm}^2/\text{h}$  permeability, within 24 h, it was indicated that the formulation has shown the sustained effect when compared with CS gel permeability i.e  $50 \mu\text{g}/\text{cm}^2/\text{h}$  in 8h. The flux of the CS NLCs-PB loaded gel was  $1.024 \mu\text{g}/\text{cm}^2/\text{h}$ . The skin deposition study of CS NLCs- PB loaded gel has shown the 2.8 folds higher than the CS gel. It was indicated that the developed formulation has the higher deposition of the CS on skin layers

The anti-psoriatic effect of the CS NLCs-PB loaded gel on imQ induced mouse was produced by the reduction of PASI (Erythema, scaling, thickness of skin and ear). The histological studies of treatment groups revealed that the reduction of acanthosis, hyperkeratosis and inflammatory mediators. Furthermore, the reduction of TNF- $\alpha$  levels and induction of the IL-10 levels in the ImQ induced mice model was confirmed by the ELISA test. The antioxidant property of the novel gel was confirmed by the study of increment levels of catalase, SOD, GSH and decreased levels of the MDA. In addition to that the splenomegaly also decreased upon topical

application of the developed gel. It was concluded the novel composition containing CS NLCs-PB loaded gel was successfully reduced the symptoms associated with the psoriasis and even it shown the dual pharmacological effect by the reduction of TNF- $\alpha$  and induction of IL-10 levels. The overall research study concluded that the developed formulation has the potential role in the management of the psoriasis without any side effects, which was confirmed from the preclinical study conducted on mouse. However, to explore it further for human use clinical studies need to be performed. Further the research entailed that the topical nanoformulation containing CS and PB, are suitable in the treatment of psoriasis, upon loading into the gel.

In addition, the challenges such as control on size, ease of production at large scale as well as clinical correlation with preclinical results are required to be addressed. Nevertheless, this formulation has given a new avenue for the researchers working in the area of psoriasis.

# CHAPTER 7



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**Publications/ patents/ copyrights etc. Accepted/ published/ applied so far against any of the approved objectives**

**Appendix**

S.No.	Patent/Publication/book-chapter/webinar/event	Title	Journal name/IPR India	Status
<b>Copyrights</b>				
1	Copyright	Formulation and evaluation of nano lipid based gel of chrysin and probiotic for psoriasis	IPR	Granted
<b>Patents</b>				
2	Patent	A novel lipid based gel composition comprising of 5, 7 di-hydroxy flavone and <i>Bifidobacterium infantis</i> 35624	IPR India.	Patent No:202011031511
<b>Events</b>				
3	Event	Training on The care and Use of Laboratory Animals in Accordance with Ethical Guidelines (Feb-03-04, 2023)	Lovely Professional University	Program Facilitator
4	3 <sup>rd</sup> international symposium women and girls in Science (2023)	Chrysin: A small molecule loaded in nanostructured lipid carriers for psoriasis	KIET group of institutions	Presentee
5	3 <sup>rd</sup> international conference (ICP-2022)	Development and characterization of nanostructured lipid carriers of chrysin	Lovely Professional University	Presentee
6	National symposium on “Translational research and future Pharmaceuticals” (2022)	Nanostructured lipid carriers containing 5,7 dihydroxy flavone for psoriasis	Organized by JSS Ooty	<b>1<sup>st</sup> Prize under the category poster Presentation Competition</b>
7	2 days International Conference on Advances in Pharmaceutical and Health sciences (APHS-2021) International Conference on Advances in Pharmaceutical and Health sciences (2021)	Development and validation of reverse phase high-performance liquid chromatography method for chrysin	Organized by KIET School of Pharmacy, KIET Group of Institutions, Delhi –NCR, Ghaziabad in collaboration with CSIR Indian Institute of Toxicological Research Luck	<b>1<sup>st</sup> Prize under the category of Oral Presentation Competition</b>

			now, India.	
<b>Research articles</b>				
8	Research article	Validated RP-HPLC method for Estimation of Chrysin in Bulk Form and Nanostructured lipid carriers for Topical Applications	Nano science and nanotechnology Asia	Published
9	Research article	Formulation of chrysin loaded nanostructured lipid carriers using Box Behnken Design, its characterization and antibacterial evaluation alone and in presence of probiotics co-loaded gel	Journal of Drug Delivery Science and Technology JDDST-D-23-00093R1	Published
10	Research article	“Analytical Quality Design Based UV-visible Spectrophotometer Method Development and Validation for Quantification of Chrysin in Bulk and Nanostructured Lipid Carriers”	Annals of Biology	Accepted
11	Research article	Antipsoriatic evaluation of a novel gel containing Chrysin nanostructured lipid carrier and probiotic in imiquimod induced mice model	Naunyn-Schmiedeberg's Archives of Pharmacology	Under review
<b>Book chapter</b>				
12	Book chapter	Nano medicine based approaches for management of psoriasis	Taylor and Francis Journal	Under review
<b>Review article</b>				
13	Review article	Nano-phyto Pharmaceuticals: An alternative road to psoriasis treatment	Journal of Emerging Technologies and Innovative Research JETIR December 2021, 10Volume 8, Issue12	Published
<b>Symposium</b>				
14	E-Symposium	2 <sup>nd</sup> International E-Symposium on Women & Girls in Science	Organized by KIET School of Pharmacy, KIET Group of Institutions, Delhi –NCR,	Delegate

			Ghaziabad in collaboration with Health Pram	
<b>Webinar</b>				
15	Webinar	International conference on current scenario in advancements of pharmaceutical education and research: challenges and prospects	Organized by Swami Vivekananda college of pharmacy. Banur, Punjab.	Delegate
16	Webinar series no:2	Lipid Excipients: For solubility & oral bioavailability-enhancement	Organized by Gattefosse	Delegate
17	Webinar	Importance of the particle Characterization and Zeta potential in Pharmaceutical	Lovely Professional University	Delegate
18	Short term course	Short term course on innovative practise in scientific writing and communication	Lovely Professional University	Delegate





Extracts from the Register of Copyrights



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Review Date: 21/09/2023

- 1. रजिस्ट्रार नंबर/Registrar Number
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TITLE: FORMULATION AND EVALUATION OF NANO LIPID-BASED DELIVERY SYSTEM FOR PROBIOTIC FOR PROBIOSIS
AUTHOR: SHAIK SAHANA PARVEEN, LOVELY PROFESSIONAL UNIVERSITY, ALANDIAR DELHI-GT ROAD, BHAGWARA, PUNJAB-146411, INDIA
DE. SHEETU WADUWA, LOVELY PROFESSIONAL UNIVERSITY, ALANDIAR DELHI-GT ROAD, BHAGWARA, PUNJAB-146411, INDIA
DE. SACHIN KUMAR SINGH, LOVELY PROFESSIONAL UNIVERSITY, ALANDIAR DELHI-GT ROAD, BHAGWARA, PUNJAB-146411, INDIA
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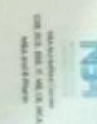
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This is to certify that Ms. Shaik Rahana Praveen, D/o Sh. Shaik Mahabub Subhani participated as Program Facilitator in Training on The Care and Use of Laboratory Animals in Accordance with Ethical Guidelines organized by Lovely Professional University w.e.f. February 03, 2023 to February 04, 2023.

Secretary, IPGA Local Chapter  
Phagwara

Head  
Human Resource Development Center

Registrar  
Lovely Professional University



3<sup>rd</sup> International Symposium

On

# Women & Girls in Science

In collaboration with APTI Women's Forum

11<sup>th</sup> February, 2023 (Saturday)

## CERTIFICATE OF APPRECIATION

This is to certify that Dr./Mr./Ms. Shweta Raksha Pareek

from School of Pharmaceutical Sciences, Lovely Professional University, Punjab

has presented (Oral/Poster) a paper titled

Chaperin : A small molecule based in nanostructured lipid carriers for protein

in 3<sup>rd</sup> International Symposium on "Women & Girls in Science"

organized by KIET School of Pharmacy, Delhi-NCR, Ghaziabad.

*Shweta*

Dr. Vandana Patravale  
National Convener  
APTI Women's Forum

*W.P.*

Dr. Vekhalil M. Patel  
Organizing Secretary

*W.P.*

Prof. (Dr.) K. Nagarajan  
Convener & Principal  
KIET School of Pharmacy



Organized by:  
Dept. of Pharmaceutics  
(DST-FIST Sponsored)



# CERTIFICATE OF ACHIEVEMENT

Awarded to

*Mr. Dhritik Rohana Kevien*

for securing the FIRST place in the poster presentation titled

"NANOSTRUCTURED LIPID PARTICLES CONTAINING S.T.D"

"HYDROXY FLAVONE FOR ROSAHSIS"

conducted during the two day  
National Symposium on "Translation Research and Future Pharmaceuticals"  
on 04 & 05 November 2022, at JSS College of Pharmacy, Ooty.

*[Signature]*

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CHAIRPERSON  
Principal  
JSSCP, Ooty

*[Signature]*

Dr. Monica Gubali

CHAIRPERSON  
Registrar  
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for his/her outstanding performance in Oral Presentation & achieved **1st Prize** under the category of  
in 2 Days International Conference on **Advances in Pharmaceutical and Health Sciences (APHS 2021)**  
during 07<sup>th</sup> - 08<sup>th</sup> October 2021

organized by KIET School of Pharmacy, KIET Group of Institutions, Delhi-NCR, Ghaziabad  
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*Lovely Professional University*

has participated as **Delegate** in 2 Days International Conference on  
**Advances in Pharmaceutical and Health Sciences**  
**(APHS 2021)**

during 07<sup>th</sup> - 08<sup>th</sup> October 2021

organized by KIET School of Pharmacy, KIET Group of Institutions, Delhi-NCR, Ghaziabad  
in collaboration with **CSIR-Indian Institute of Toxicological Research Lucknow, India.**

**Dr. Vaishali Manikrao Patil**  
Organizing Secretary  
Associate Professor-KSOP, Ghaziabad

**Dr AB Pant**  
Convener  
CSIR-IITR, Lucknow, India

**Prof. (Dr.) K. Nagarajan**  
Convener  
Principal-KSOP, Ghaziabad



# Women & Girls in Science

In collaboration with HealthPrax  
12<sup>th</sup> February, 2022 (Saturday)



## 2<sup>nd</sup> International E-Symposium

On

### Certificate of Participation

This is to certify that

**SHAIK RAHANA PARVEEN**

has attended 2<sup>nd</sup> International e-Symposium on "Women & Girls in Science"  
organized by KIET School of Pharmacy, Delhi-NCR, Ghaziabad.

**Dr. Wojna Sah Jain**  
CEO, HealthPrax

**Dr. Vaisakh M. Pardi**  
Organizing Secretary

**Prof. (Dr.) K. Nagarajan**  
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**DEPARTMENT OF PHARMACEUTICAL SCIENCES**  
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This is to certify that Prof./Dr./Mr./Ms. **SHAIK RAHANA PARVEEN**.....

from **LOVELY PROFESSIONAL UNIVERSITY**.....attended

as **DELEGATE** in Two Days program held on 25-26th March 2021 through online mode.

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Patron

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**Prof. Harish Dureja**  
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UNIVERSITY**

Balawala, Dehradun, Uttarakhand, India

*Certificate of Participation*

This is to Certify that *Dr./ Mr./ Ms* \_\_\_\_\_ Shaik Rahana Parveen

*has participated in the webinar on "Data Analytic Tools for Pharmaceutical and  
Chemical Sciences Researchers" conducted on Wednesday, March 10<sup>th</sup>, 2021.*

Secretary (RDC)

Director (SPST)

Vice Chancellor (O)



INTERNATIONAL CONFERENCE  
On

**CURRENT SCENARIO IN ADVANCEMENTS OF PHARMACEUTICAL EDUCATION &  
RESEARCH: CHALLENGES & PROSPECTS**  
SVCPCON-2020  
29<sup>th</sup>-31<sup>st</sup> December 2020

Organized by

**SWAMI VIVEKANAND COLLEGE OF PHARMACY, BANUR, PUNJAB**

*Certificate of Participation*

This is to certify that Dr/Mr/Ms. Shaik Rahana Parveen of Lovely Professional University participated as Delegate during the conference held on 29<sup>th</sup>-31<sup>st</sup> December 2020

Mr. Suresh Prakash  
Organizing Secretary

Dr. Parveen Sarvag  
Conference Chair

Mr. Akshay Singh  
President

Mr. Ashwani Singh  
Chairman

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**Shaik Rahana Parveen**  
Lovely Professional University, Punjab

attended the webinar on

**Importance of Particle Characterization  
and Zeta Potential in Pharmaceutical**

Date: July 22, 2021  
Time: 11:30 to 12:30 hours

**Contents**

The training provided the essential knowledge for:

- ▶ Role of particle size in pharmaceutical development
- ▶ Discussing the impact of particle size in different diseases
- ▶ Brief about the basics of Dynamic Light Scattering
- ▶ Outlining the ideal Light Scattering Instrument

Date of issue: July 23, 2021

Dr. Rishi Gupta  
Application Specialist - Characterization Division  
Anton Paar India Pvt. Ltd.





Certificate No. 229173

## Certificate of Participation

This is to certify that **Ms. Shaik Rahana Parveen D/o Shaik Mahabu Subhani** participated in **Short Term Course on Innovative Practices in Scientific Writing and Communication 2.0** organized by Lovely Professional University w.e.f. **July 26, 2021 to August 02, 2021** (7 days) and obtained "B" Grade.

Date of issue : 02-08-2021  
Place : Sangrur (Punjab), India



Prepared by  
(Administrative Officer-Records)

Dr. Bimlesh Kumar  
Associate Professor  
Department of Pharmaceutical Sciences  
Lovely Professional University

Dr. Sunaina Ahuja  
Head-Human Resource  
Development Center  
Lovely Professional University

Dr. Monica Gulati  
Senior Dean  
Faculty of Applied Medical Science  
Lovely Professional University



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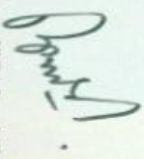
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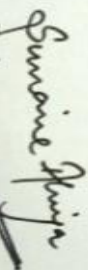


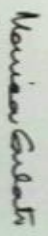
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This is to certify that **Ms. Shaik Rahana Praveen**, D/o Sh. Shaik Mahabub Subhani participated as Program Facilitator in **Training on The Care and Use of Laboratory Animals in Accordance with Ethical Guidelines** organized by Lovely Professional University w.e.f. February 03, 2023 to February 04, 2023.

  
Secretary, IPGA Local Chapter  
Phagwara

  
Head  
Human Resource Development Center

  
Registrar  
Lovely Professional University



Serial No. 3ICP2022 1465

# Certificate of Participation


This is to certify that Prof./Dr./Mr./Ms. Shaik Rabana Parveen has successfully participated as Delegate & Presented Poster/Oral Presentation on Development and Characterisation of Nanostructured lipid carriers of drug in the 3<sup>rd</sup> International Conference of Pharmacy (ICP-2022) on the Theme of "Practice, Promotion & Publication of Innovation : A Way of Transforming Health" held on 09<sup>th</sup> & 10<sup>th</sup> November 2022 organized by School of Pharmaceutical Sciences in a collaboration with Indian Pharmaceutical Association (IPA) at Lovely Professional University, Punjab.


  
Mr. Suresh Khanna  
National Hon. Gen  
Secretary, IPA

  
Dr. Birmlesh Kumar  
Organizing Secretary

  
Dr. T.V. Naryana  
National President  
IPA

  
Dr. Monica Gulati  
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Sri Herbasia Biotech

  
Tishk International University, Iraq

## Reserach article







Journal of Drug Delivery Science and  
Technology

Volume 84, June 2023, 104411



# Formulation of chrysin loaded nanostructured lipid carriers using Box Behnken design, its characterization and antibacterial evaluation alone and in presence of probiotics co-loaded in gel

[Shaik Rahana Parveen](#)<sup>a</sup>, [Sheetu Wadhwa](#)<sup>a</sup>  , [Molakpogu Ravindra Babu](#)<sup>a</sup>, [Sukriti Vishwas](#)<sup>a</sup>, [Leander Corrie](#)<sup>a</sup>, [Ankit Awasthi](#)<sup>a</sup>, [Farhan R. Khan](#)<sup>b</sup>, [Maha M. Al-Bazi](#)<sup>c d</sup>, [Nahed S. Alharthi](#)<sup>e</sup>, [Faisal Alotaibi](#)<sup>f</sup>, [Gaurav Gupta](#)<sup>g h</sup>, [Narendra Kumar Pandey](#)<sup>a</sup>, [Bimlesh Kumar](#)<sup>a</sup>, [Popat Kumbhar](#)<sup>i</sup>, [John Disouza](#)<sup>i</sup>, [Monica Gulati](#)<sup>a j</sup>, [Jayanthi Neelamraju](#)<sup>k</sup>, [Ratna Sudha Mademudi](#)<sup>k</sup>, [Kamal Dua](#)<sup>j l m</sup>, [Sachin Kumar Singh](#)<sup>a j</sup>  

### RESEARCH ARTICLE

## Validated RP-HPLC Method for Estimation of Chrysin in Bulk Form and Nanostructured Lipid Carriers for Topical Application

Shaik Rahana Parveen<sup>1</sup>, Sheetu Wadhwa<sup>1,\*</sup>, Sachin Kumar Singh<sup>1</sup>, Bhupinder Kapoor<sup>1</sup>, Pooja Rani<sup>1</sup> and Sukriti Vishwas<sup>1</sup>

<sup>1</sup>School of Pharmaceutical Sciences, Lovely Professional University, Punjab-144411, India

**Abstract: Background:** *Chrysin*, a flavonoid, occurs naturally in plants and possesses many pharmacological actions, but there is a lack of suitable analytical methods for its estimation.

**Objective:** To develop a simple analytical method and validate it for the estimation of *chrysin* using reverse phase high-performance liquid chromatography (RP-HPLC).

**Methods:** Isocratic elution was carried out in methanol and 0.1% v/v formic acid in a 70:30 ratio using a C-18 reverse-phase column. The flow rate was set to 1 mL min<sup>-1</sup> and the detection wavelength at 268 nm. As per ICH Q2 (R1) guidelines, the developed method was validated in terms of accuracy, precision, system suitability, and robustness.

**Results:** The retention time of *Chrysin* was found at 10.269 min. In the concentration range of 2-10 µg/mL, the developed method was linear with a regression coefficient ( $R^2$ ) value of 0.998. The mean percentage recovery of *chrysin* was found within 95-105% at all three levels, which confirms that the developed method was accurate. Moreover, the % RSD was found to be less than 2% confirming that the developed method was precise. The limit of detection and limit of quantification were found to be 0.071 and 0.217 µg/mL. Moreover, the validated method was robust with no significant changes in response to variation in flow rate, mobile phase composition, wavelength, and different lots of columns.

#### ARTICLE HISTORY

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Accepted: December 29, 2022

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## Acceptance letter

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E-mail: bajdass@gmail.com

Telephone: 01662-237530; Mobile 092551-26155; ISSN: 0970-0153

Ref. No. AOB/2022/03

Dated: 03.12.2022


Dear Dr. Sheetu Wadhwa/Sachin Kumar Singh,

The paper entitled "**Analytical Quality Design Based UV-visible Spectrophotometer Method Development and Validation for Quantification of Chrysin in Bulk and Nanostructured Lipid Carriers**" by "**Shaik Rahana Parveen, Sheetu Wadhwa, Sachin Kumar Singh, Vancha Harish Leander Corrie and Ankit Awasthi**" has been received for publication in the Annals of Biology.

The paper has been given a MS No. 2023/09 which should be quoted while making future correspondence about the paper.

I am happy to inform you that subject to editorial corrections the above referred manuscript has been accepted for publication in Annals of Biology. Every effort will be made to accommodate it in coming issue of Annals of Biology.

Yours sincerely,

  
(Editor)

Dr. Sheetu Wadhwa/Sachin Kumar Singh,  
School of Pharmaceutical Sciences,  
Lovely Professional University,  
Phagwara - 144 411

## Review article

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**JOURNAL OF EMERGING TECHNOLOGIES AND  
INNOVATIVE RESEARCH (JETIR)**

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# Nano phyto-pharmaceuticals: An alternative road to Psoriasis treatment

Shaik Rahana Parveen<sup>1</sup>, Arya K.R<sup>1</sup>, Sheetu wadhwa<sup>1</sup>, Bhupinder Kapoor<sup>1</sup>  
[shaikrahanaparveen@gmail.com](mailto:shaikrahanaparveen@gmail.com), [sheetu.21001@lpu.co.in](mailto:sheetu.21001@lpu.co.in), [arya.jithu07@gmail.com](mailto:arya.jithu07@gmail.com),  
[bhupinder.14146@lpu.co.in](mailto:bhupinder.14146@lpu.co.in)

<sup>1</sup>School of Pharmaceutical Sciences, Lovely Professional University, Phagwara-144411, Punjab, India,  
+917893461860; Fax: +91 1824501900

**Abstract:** Nano phyto-pharmaceuticals are the current focused study for the psoriasis management in an alternative manner. Psoriasis is a skin disease that lasts for a long time. It is a multifactorial skin disease, making it impossible to provide a suitable solution to date. Since psoriasis is an autoimmune condition, proper treatment is more crucial than just providing medicine. Long therapy durations of exciting synthetic drugs are associated with a higher incidence of side effects. These side effects can be overcome by using alternative strategy is Nano phyto-pharmaceuticals. Many psoriasis patients find that taking vitamins, applying oils, extracts of herbals on skin, and food supplements will benefit them to soothe irritated skin and aid in skin clearing. The available electronic data analysis has been performed for the thorough assessment of widely used herbals for psoriasis. Herbal extracts, oils and active phyto constituents have shown to be useful in preclinical and clinical trials and are prescribed as a psoriasis auxiliary therapy. A few of herbal extracts or phyto constituents are available in a variety of dosage forms, including pills, capsules, soft gelatin capsules,

**CENTRAL ANIMAL HOUSE FACILITY (CAHF)**  
Lovely Institute of Technology (Pharmacy), Lovely Professional University  
Ludhiana- Jalandhar G.T. Road, Phagwara (Punjab), 144411  
Registration Number -954/PO/ReReBiBt/S/06/CPCSEA

---

**CERTIFICATE**

This is to certify that the project titled "*Evaluation of Antipsoriatic potential of individual Chrysin, probiotic and its combination loaded in nanostructured lipid based carriers gel on experimental animals.*"  
(Thesis title: *Development and evaluation of topical nanoformulation of Chrysin and probiotic for psoriasis*)" has been approved by the IAEC.

Name of Principal Investigator: Dr. Sheeta

IAEC approval number: LPU/IAEC/2021/85

Date of Approval: 24<sup>th</sup> September 2021

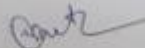
Animals approved: 66 Swiss albino mice, either sex

Remarks if any: - NA



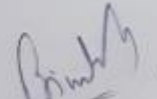
**Dr. Monica Gulati**

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**Dr. Navneet Khurana**

Scientist from different discipline



**Dr. Bimlesh Kumar**

Scientist In-Charge of Animal House,  
Member Secretary IAEC