DESIGN, DEVELOPMENT AND CHARACTERIZATION OF OCULAR NANOSTRUCTURED LIPID CARRIERS OF 5-FLUOROURACIL FOR THE TREATMENT OF DIABETIC RETINOPATHY

A

Thesis

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By

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DECLARATION

I, hereby declared that the presented work in the thesis entitled "Design, development and characterization of ocular nanostructured lipid carriers of 5-fluorouracil for the treatment of diabetic retinopathy" 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 School of Pharmaceutical Sciences of 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 "Design, development and characterization of ocular nanostructured lipid carriers of 5-fluorouracil for the treatment of diabetic retinopathy" submitted in fulfillment of the requirement for the reward of degree of Doctor of Philosophy (Ph.D.) in the School of Pharmaceutical Sciences, is a research work carried out by Deep Shikha Sharma, 41700243, is bonafide record of 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 main objective of the present study is to develop surface modified 5-Fluorouracil (5-FU) nanostructured lipid carriers (NLCs) by ocular route for the management of Diabetic Retinopathy (DR). The solid lipid, liquid lipid, surfactant and cosurfactant were screened on the basis of solubility studies. The modified melt emulsification method was used to prepare the 5-FU-NLCs and the formulation was optimized by Box-Behnken design (BBD) and ternary phase diagram (TPD) was created. 5-FU-NLCs were surface modified by positively charged biopolymer chitosan (CS) to improve permeability of 5-FU at the desired site i.e. retina. The optimized batch 5-FU-NLCs and final batch CS-5-FU-NLCs were evaluated for particle size, zeta potential, polydispersity index, percentage entrapment efficiency and percentage drug loading. Drug and excipient compatibility study was performed by Differential Scanning Calorimetry (DSC) and Fourier transform infrared (FTIR) spectroscopy. Scanning electron microscopy (SEM) and Transmission electron microscopy (TEM) analysis were done to observe surface morphology. *In vitro* drug release and *ex vivo* permeation studies were performed to check drug release in CS-5-FU-NLCs and compared with 5-FU solution and 5-FU-NLCs. Hen's Egg Test-Chorioallantoic Membrane (HET-CAM) model was used to ensure ocular non-irritant nature of CS-5-FU-NLCs. Further, CS-5-FU-NLCs were examined for antiangiogenic activity and its Vascular Endothelial Growth Factor (VEGF) targeting efficiency *in vivo* by CAM and DR induced rat model.

The solubility study results revealed that 5-FU was more soluble in glyceryl monostearate (GMS, solid lipid), labrafil M 2125CS (LMCS, liquid lipid), tween 80 (surfactant), transcutol HP (cosurfactant). The BBD was applied to optimize the formulation variables affecting NLCs. The optimized batch of 5-FU-NLCs exhibited nano size range (131.10 nm), optimum zeta potential (-16.00 mV), narrow size distribution (0.260 polydispersity index) and higher % entrapment efficiency (81.40 %) and optimum % drug loading (16.25 %). The final batch CS-5-FU-NLCs exhibited nano size range (163.20 nm), narrow size distribution (0.28 polydispersity index), optimum zeta

potential (21.40 mV), and higher % entrapment efficiency (85.00 %) and optimum % drug loading (17.00 %). DSC and FTIR shown encapsulation of 5-FU inside the matrix of lipids. SEM and TEM revealed surface modification of CS-5-FU-NLCs. *In vitro* drug release and *ex vivo* permeation study confirmed higher and sustained drug release in CS-5-FU-NLCs as compared to 5-FU solution and 5-FU-NLCs. HET-CAM model ensured the non irritant nature of CS-5-FU-NLCs. *In vivo* ocular studies of CS-5-FU-NLCs confirmed antiangiogenic effect of 5-FU by CAM model and diabetic retinopathy induced rat model, which indicated successful delivery of 5-FU to the retina by improving its permeability at desired site.

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Deep Shikha Sharma

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LIST OF ABBREVIATIONS

Symbol/ Abbreviations	Full form
%	Percentage
°C	Degree Centigrade
µL	Microlitre
µg	Microgram
ng	Nanogram
α	Alpha
AIOS	All India Ophthalmological Society
ARI	Aldose Reductase Inhibitors
AGE	Advanced Glycation End
ACE	Angiotensin-Converting Enzyme
ACE-2	Angiotensin-Converting Enzyme-2
ARB	Angiotensin Receptor Blocker
AT-I	Angiotensin-I
AT-II	Angiotensin-II
ANOVA	Analysis of Variance
API	Active Pharmaceutical Ingredients
ADA	American Diabetes Association
AMD	Age-related Macular Degeneration
β	Beta
BBD	Box-Behnken Design
BRB	Blood Retinal Barrier
BGL	Blood Glucose Level
BCS	Biopharmaceutical Classification System
BP	Blood Pressure

BDNF	Brain-Derived Neurotrophic Factor
CS	Chitosan
CC	Calibration Curve
CVS	Cardio Vascular System
CS	Chitosan
CMC	Critical Micelle Concentration
CS-5-FU-NLCs	Chitosan-5-Fluorouracil-Nanostructured Lipid Carriers
CPCSEA	Committee for the Purpose of Control and Supervision of Experiments on Animals
CDC	Center for Disease Control and Prevention
CNV	Choroidal Neovascularization
cm	Centimeter
cm^2	Centimeter square
cm^{-1}	Centimeter inverse
CMCM	Capmul MCM
Conc.	Concentration
CAM	Chorioallantoic Membrane
CT	Centrifuge Tube
COX-1	Cycloxygenase 1
COX-2	Cycloxygenase 2
DR	Diabetic Retinopathy
DME	Diabetic Macular Edema
DM	Diabetes Mellitus
DoE	Design of Experiment
DSC	Differential Scanning Calorimetry
DESP	Diabetic Eye Screening Programme

DRSW	Diabetic Retinopathy Screening Service Wales
DRS	Diabetic Retinopathy Screening
% EE	Percentage Entrapment Efficiency
Eg	Example
ERK	Extracellular signal Regulated Kinase
ERG	Electroretinography
ELISA	Enzyme-Linked Immunoassay
FDC	Franz Diffusion Cell
5-FU	5-Fluorouracil
5-FU-NLCs	5-Fluorouracil-Nano structured Lipid Carriers
FTIR	Fourier Transform Infrared Spectroscopy
Fig.	Figure
γ	Gamma
g	Gram
GMS	Glyceryl Mono Stearate
GR	Glutathione Reductase
GSH	Glutathione
GH	Growth Hormone
GEN	Genistein
GHI	Growth Hormone Inhibitor
GHIH	Growth Hormone Inhibiting Hormone
GNP	Gold Nanoparticles
GFAP	Glial Fibrillary Acidic Protein
H_2O_2	Hydrogen Peroxide
HET-CAM	Hen's egg test-Chorioallantoic membrane model
HO-1	Heme Oxygenase-1

h	Hour
HPLC	High Performance Liquid Chromatography
HG	High Glucose
HuR	Human Antigen R
HETP	Height Equivalent to Theoretical Plate
HDL	High Density Lipoproteins
HQC	Higher Quantification Concentration
HLB	Hydrophilic Lipophillic Balance
ICH	International Conference on Harmonization
IDF	International Diabetes Federation
IS	Irritation Score
IL-1β	Interleukin-1β
ITR	Itraconazole
IOP	Intraocular Pressure
IAEC	Institutional Animal Ethics Committee
ITR-NLCs	Itraconazole-Nanostructured Lipid Carriers
LOD	Limits of Detection
LOQ	Limits of Quantification
Log P	Partition Coefficient
LQC	Lower Quantification Concentration
LMCS	Labrafil M 2125CS
LE	Left Eye
LC	Liquid Crystals
LDL	Low-Density Lipoproteins
M.R.T	Mean Retention Time
mg	Milligram

min	Minute
mL	Milliliter
MQC	Medium Quantification Concentration
MMP9	Matrix Metalloproteinase-9
Myr	Myriocin
Myr-SLN	Myriocin-Solid Lipid Nanoparticles
MMP	Matrix Metalloproteinases
MCP1	Monocyte Chemo-tactic Protein1
MAP	Mitogen Activated Protein
mAb	Monoclonal Antibody
Nrf2	Nuclear factor erythroid 2-related Factor 2
NaOH	Sodium Hydroxide
NPDR	Non-Proliferative Diabetic Retinopathy
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NDDS	Novel Drug Delivery System
ng	Nanogram
NLCs	Nanostructured Lipid Carriers
NFkβ	Nuclear Factor Kappa beta
nm	Nanometer
NPs	Nanoparticles
NSAIDS	Non-Steroidal Anti-Inflammatory Drugs
NDESP	National Diabetic Eye Screening Programme
OPA	Ortho-Phosphoric Acid
PDI	Polydispersity Index
PC	Partition Coefficient

PPAR-α	Peroxisome Proliferator-Activated Receptor-alpha
PEA	Palmitoylethanolamide
PRP	Pan Retinal Photocoagulation
PDR	Proliferative Diabetic Retinopathy
PEG	Polyethylene Glycol
PG	Propylene Glycol
PS	Particle Size
PBS	Phosphate Buffer Saline
PFA	Paraformaldehyde
PKC	Protein Kinase C
PKC 1	Protein Kinase C 1
PKC 2	Protein Kinase C 2
RE	Right Eye
RPE	Retinal Pigment Epithelium Cells
RCE	Retinal Capillary Endothelial Cells
ROSp	Reactive Oxygen Species
ROS	Retinal Oxidative Stress
RBX	Ruboxistaurin
RGCs	Retinal Ganglion Cells
RP-HPLC	Reverse Phase High Performance Liquid Chromatography
RBPs	RNA-Binding Proteins
RNA	Ribo Nucleic Acid
RNAi	Ribo Nucleic Acid Interference
RAAS	Rennin-Angiotensin-Angiotensinogen System
RPE	Retinal Pigment Epithelial

rpm	Rotations Per Minute
RH	Relative Humidity
RT	Retention Time
R^2	Coefficient of determination
rVEGF	Recombinant Vascular Endothelial Growth Factor
RGD	Arginine-glycine-aspartic acid
% RSD	Percentage Relative Standard Deviation
RP	Retinitis Pigmentosa
S.D	Standard Deviation
SLNs	Solid Lipid Nanoparticles
SEM	Scanning Electron Microscope
STF	Simulated Tear Fluid
STZ	Streptozotocin
SD	Sprague Dawley
SV	Simian Virus
TIDM	Type 1 Diabetes Mellitus
T2DM	Type 2 Diabetes Mellitus
TrkB	Tropomyosin Related Kinase B
TNF-α	Tumor Necrosis Factor-alpha
T80	Tween 80
TEM	Transmission Electron Microscope
Temp.	Temperature
THP	Transcutol HP
TPD	Ternary Phase Diagram
TfR	Transferrin Receptor
UK	United Kingdom

UKPDS	United Kingdom Prospective Diabetes Study
VEGF	Vascular Endothelial Growth Factor
VIF	Variance Inflation Factor
VEGFR-2	Vascular Endothelial Growth Factor-2
wAMD	Wet Age-related Macular Degeneration
XRD	X Ray Diffraction
ZP	Zeta Potential

1. INTRODUCTION

Diabetes Mellitus (DM) is a metabolic disorder that occurs due to the elevated blood glucose level (BGL). DM is broadly categorized into two types-Type-1 Diabetes Mellitus (T1DM) and Type-2 Diabetes Mellitus (T2DM). Over 422 million people worldwide have diabetes and almost 1.6 million deaths are due to diabetes every year. The global prevalence rate of diabetes is more than 8.5% of the population over last 18 years. The number of diabetic people is expected to increase upto 700 million by 2045. There are some diabetes related complications which may leads to nerve damage i.e. neuropathy, renal damage i.e. nephopathy, eye damage i.e. diabetic retinopathy (DR), foot damage, alzheimer's disease, etc. [Zaccardi *et al.*, 2016]**.**

As per International Diabetes Federation (IDF) 2021, about 537 million adults aged 20-79 years worldwide (9.3% of all adults in this age group) have diabetes and it is estimated that out of which 79.4% live in low and middle income countries. Based on the 2021 estimates, by 2045 a projected 783 million adults aged 20-79 years, will be living with diabetes [http://diabetesatlas.org/en].

Diabetic retinopathy (DR) is a common comorbidity among patients having a prolonged history of DM. It damages retinal blood vessels and retinal nerves and is considered as a leading cause of vision loss, or complete blindness among the diabetic population worldwide [Sharma *et al.*, 2021]. DR is classified into two types: proliferative diabetic retinopathy (PDR) and non-proliferative diabetic retinopathy (NPDR). In PDR, neovascularization (abnormal growth of the blood vessels) of ocular blood vessels takes place, which leads to sudden vision loss. NPDR is further divided into three categories, i.e. mild, moderate, and severe NPDR. In mild NPDR, microaneurysms (balloon-like swelling) occur that damage the small retinal blood vessels. In moderate NPDR, blood vessels of the retina get blocked causing a deficiency in the supply of oxygen and nutrients required for normal retinal functioning. In severe NPDR, many

blood vessels of the retina get blocked and severe oxygen and nutrient depletion happens at the level of the retina [Wilkinson *et al.*, 2003].

Presence of anatomical and physiological retinal eye barriers poses challenge for the treatment of DR. Success of any treatment of DR depends on the targeting of the drug to the posterior segment of eye i.e. retina by crossing or by passing ocular barriers for the management of DR. Blood retinal barriers (BRB) do not allow the movement of drugs from blood into the posterior part of the eye [Kim *et al.*, 2009].

In particular, ocular diseases have been treated by two primary modalities, i.e. topical routes and invasive routes. From the last few decades, topical drops have been a mainstay, however it lacks in patient compliance. About 33% of the patients undergoing therapy have been reported to discontinue their therapeutic schedule after one year of eye drops administration [Reardon *et al.,* 2011].

The introduction of intraocular injections has provided the first effective route for eye therapy. Moreover, the approval of ranibizumab and aflibercept for the treatment of wet age-related macular degeneration (wAMD) has provided a breakthrough in intraocular drug delivery [Rofagha *et al.*, 2013].

The discovery of implantable intraocular devices such as Vitrasert, Retisert, Ozurdex, and Iluvein has been able to provide long term residence within the eye [Haller *et al*, 2010; Callanan *et al.*, 2008].

Anti-VEGF drugs like ranibizumab, aflibercept, bevacizumab, and pegaptanib play a very important role in DR treatment. These drugs prevent blindness and improve vision in diabetic patients, but can cause serious complications of eyes like impaired wound healing, retinal detachment, endophthalmitis, hypertension, proteinuria, and increased risk of cardio vascular system (CVS) diseases [Sharma *et al*., 2020a].

Ocular conventional systems include ophthalmic drops, suspensions, emulsions, and ointments that are being used for the treatment of ocular diseases. Mostly topically applied drug entities are removed and washed off from the eyeball by mechanism of lacrimation, tear dilution, and tear turnover ultimately causing low bioavailability of drugs, Therefore, about 5% of administered or topically applied drug at ocular site enters the eye [Loftsson *et al*., 2007]. The major reasons for the moderate success of

existing therapies including protein binding, reduction in drug's concentration on instillation, less space at ocular site, invasive process, and high cost of treatment [Edelhauser *et al*., 2010]. The challenges of the afore mentioned existing conventional dosage forms suggest to rethink on the need for non-invasive, effective, and economical delivery systems. In recent years novel drug delivery system (NDDS) have emerged as a potential tool that can deliver the drug effectively to retina over-coming the challenges associated with conventional as well as surgical approaches [Fangueiro *et al.*, 2015].

Several nanocarriers have been explored to prevent diabetes related complications [Attia Shafie *et al.*, 2013]. For example, solid lipid nanoparticles (SLNs) [Takte *et al.*, 2019], polymeric nanoparticles (NPs) [Lu *et al*., 2014], gold nanoparticles [Kim *et al.*, 2009], nanostructured lipid carriers (NLCs) [Selvaraj *et al*., 2019], liquid crystals (LC) [Liu *et al.*, 2016], liposomes [Tan *et al.*, 2017], and microemulsions [Üstündağ Okur *et al*., 2020] have shown several advantages as compared to conventional drug delivery systems, such as increased surface area, improved adhesion, depot formation, enhanced biocompatibility, and controlled drug release rate. In addition, they reduce the side effects of the drugs and increase patient compliance, overcoming the challenges associated to treatments with drugs alone or, even with classic delivery systems such as low solubility in solvents, need of high doses to exhibit therapeutic effect, high toxicity, reduced half-life, aggregation, enzymatic, and chemical degradation. Drug loaded nanoparticles are able to overcome these limitations. In addition to this, they are able to offer targeted delivery to specific cells or tissues, sustained delivery of both water-insoluble drugs as well as macromolecules, and minimize side effects [Fangueiro *et al*., 2015].

In another study, palmitoylethanolamide (PEA) was found to show beneficial effects in several retinal diseases such as DR, glaucoma, etc. PEA attenuated the degree of retinal inflammation while preserving the blood-retinal barrier (BRB) in diabetic rats [Paterniti *et al*., 2015].

The development of NDDS and their ocular application has certainly provided an edge to treat DR over existing therapies. Among these, NLCs are potential drug deliv-

ery systems for treating ocular diseases. These are generally prepared using solids as well as liquid lipids. They can be applied topically to the eyes which can reduce pain as well as discomfort related to intravitreal injections. Because of the above-mentioned advantages, nowadays NLCs are getting more attention [Sharif Makhmal Zadeh *et al*., 2018].

In one of the studies, researchers repositioned itraconazole (ITR) NLCs to manage DR owing to its potent unutilized antiangiogenic effect [Selvaraj *et al*., 2019].

Platania *et al.,* (2019) developed Myriocin (Myr) loaded NLCs for the treatment of retinitis pigmentosa (RP) [Platania *et al*., 2019].

The present research work involves the repositioning of anticancer drug i.e. 5- Fluorouracil (5-FU) due to its potent unutilized antiangiogenic activity by developing NLCs to improve its permeability and ocular bioavailability which will target the pathogenesis of DR**.** 5-FU possesses antifibrotic, anticancer, and antiangiogenic effect [Zhang *et al*., 2017]. It is commercialized as an anticancer drug. However, due to its potent anti-angiogenic property, it can be a good candidate for treating DR. It is a Biopharmaceutical Classification System (BCS) class III drug, hence, it has good solubility but poor permeability [Zhang *et al*., 2017]. Thus, developing a formulation that can enhance the penetration/permeability of 5-FU in the eye and make it to reach at retinal site can be a novel and non-invasive approach to treat DR [Bukhari *et al*., 2018]. The NLCs are known to overcome such challenges. Hence, an attempt has been made to develop 5-FU NLCs for the treatment of DR. 5-FU-NLCs can reach to the retina because of the presence of lipid molecules and transport the drug in a more controlled mode for the treatment of DR [Schaub *et al*., 2018]. These NLCs can provide more availability, residence time, improved permeation of 5-FU to the targeted areas of the retina and offer non-invasive delivery via ocular route. Thus, they may improve patients' compliance, better ocular tolerability as well as cost effective dosage from. NLCs have the potential to control drug release, offer higher drug loading and good bioavailability. Hence, they could be a promising drug delivery system for ocular therapy [Beloqui *et al*., 2013]. However, the instability issues and anionic nature of NLCs have hindered their ophthalmic application. The NLCs with negative

charge have difficulty during their interaction with negatively charged corneal surface [Ban *et al*., 2017]. To overcome this problem, surface modification of NLCs with cationic polymers such as Eudragit-RS-100 [Zhang *et al*., 2014], chitosan (CS) [Selvaraj *et al*., 2019], PEG-400, stearylamine [Niamprem *et al*., 2019] have been reported. In a study reported by Niamprem *et al*., in 2019, it was noted that surface modification of NLCs by PEG-400, and stearylamine did not produce significant effect on particle size of drug. In another study, surface modification of lipophilic genistein with cationic polymer Eudragit-RS-100 was done. It led to interaction of genistein with corneal cells for longer duration and improved penetration. Thus, it was concluded that it can be effectively used for ocular route [Zhang *et al*., 2014].

Moreover, Chitosan (CS) was selected for surface modification of 5-FU-NLCs to modify the surface charge and improve mucoadhesion and ocular permeability of 5-FU. CS is the most widely used polymer because of its mucoadhesive, biocompatible, biodegradable, non-toxic, non-allergenic nature, and penetration enhancer [Lu *et al*., 2014; Wadhwa *et al*., 2009]. CS, composed of 2-amino-2-deoxy-h-d-glucan combined with glycosidic linkages, is a natural polysaccharide with positive charge, having the ability to get adhered to negatively charged mucosal surface of eye owing to its positive charge. Thus resulting in electrostatic interactions which leads to prolonged residence time at drug absorption sites [Nasr *et al*., 2015]. CS has been most widely used for topical delivery of drug to the posterior eye segment and act as absorbefacient for ophthalmic purpose in which the positive charged groups of CS were able to interact with cornea [Kean *et al*., 2010]. Surface modified NLCs have been found to be more effective in terms of amount of drug permeated through the cornea compared to NLCs [Rong *et al*., 2019].

2. REVIEW OF LITERATURE

2.1. Diabetes Mellitus (DM)

DM or Diabetes is a chronic condition in which the human body is not able to produce insulin or unable to use insulin which leads to hyperglycemia, i.e., the increase in the plasma glucose concentration (as shown in Table 1 below). Insulin is released from the β cells of the islets of langerhans of pancreas which helps in the breakdown of glucose. Hyperglycemia causes other complications like neuropathy, nephropathy, blindness, diabetes retinopathy, etc. Over 422 million people worldwide have diabetes and almost 1.6 million deaths are directly related to diabetes each year.

Table 1: Blood glucose level for diagnosis of diabetes

Normal Glucose level	Diabetes Condition	Impaired Glucose Tolerance Condition	Impaired Fasting Glucose Condition
During fasting condition plasma glucose level <5.6 mmol/L (100 mg/dL)	During fasting condition plasma glucose level ≥7.0 mmol/L (126 mg/dL)	During fasting condition plasma glucose level <7.0 mmol/L (126 mg/dL)	During fasting condition plasma glucose level is 6.1-6.9 mmol/L (110 to 125 mg/ dL)

DM is mainly of three types: Type 1, 2, and gestational diabetes. Other types are monogenic and secondary diabetes. The causes and symptoms of the afore mentioned types are shown in Table 2. [Zaccardi *et al*., 2016].

Table 2: Classification of diabetes

Type of Diabetes	Pathophysiology	Causes	Symptoms
Type 1	Autoimmune reaction of the body's immune system on the pancreas, leading to very little or no insulin production	Genetic susceptibility, viral infections, toxins, and dietary factors	Abnormal thirst, dry mouth, sudden weight loss, bedwetting, blurred vision, constant hunger, lack of energy, fatigue, constant urination
Type 2	Insufficient production of insulin and non-responsiveness of the body	Hyperglycaemia, resistant to insulin	Excessive thirst, dry mouth, slow healing wound, frequent and abundant urination, change in vision, foot ulcer, renal failure, or infection.
Gestational	Hyperglycemia during pregnancy	High blood glucose during pregnancy	High blood pressure, fetal macrosomia i.e., a large baby which causes difficulty in delivery
Monogenic diabetes	A single genetic mutation in an autosomal dominant	Neonatal DM and maturity-onset diabetes	Excessive thirst and hunger, frequent urination, drowsiness, unconsciousness
Secondary diabetes	Hormonal imbalance	Pancreatitis or Cushing's disease	Increase in thirst, frequent urination, weight loss, tiredness

2.2. Diabetes Retinopathy (DR)

In India, DR is a major complication of DM which leads to visual impairment. DR damages the retinal capillaries, blocks them and lead to loss of vision. It is a common comorbidity among patients having a prolonged history of DM. It damages retinal blood vessels and retinal nerves and is considered as a leading cause of vision loss or complete blindness in the diabetic population worldwide [Sharma *et al*., 2020a].

2.3. Classification of DR

DR is classified into two types: proliferative diabetic retinopathy (PDR) and non-proliferative diabetic retinopathy (NPDR). The classification of DR is illustrated in Fig. 1. In PDR, neovascularization (abnormal growth of the blood vessels) of ocular blood

vessels takes place, which are fragile and can break easily, leading to sudden vision loss. NPDR is further divided into three categories, i.e. mild, moderate, and severe NPDR. In mild NPDR, microaneurysms (balloon-like swelling) occur that damages the small retinal blood vessels. In moderate NPDR, blood vessels of the retina get blocked which causes deficiency in the supply of oxygen and nutrients required for normal retinal functioning. In severe NPDR, many blood vessels of the retina get blocked and severe oxygen and nutrient depletion happens at the level of the retina [Wilkinson *et al*., 2003].

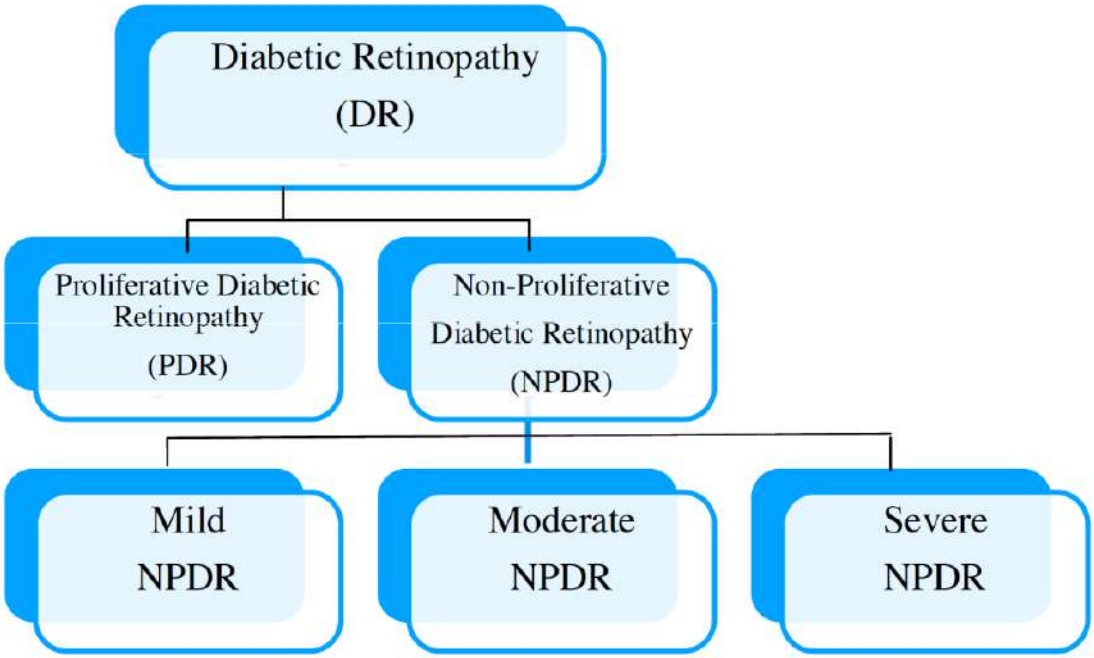


Fig. 1. Classification of diabetic retinopathy

2.4. Incidence and prevalence rate of DR

According to the IDF 2021, about 537 million adults aged 20-79 years worldwide (9.3% of all adults in this age group) have diabetes and by 2045, 783 million will have diabetes [http://diabetesatlas.org/en].

According to the IDF 2019, about 463 million adults aged 20-79 years worldwide (9.3% of all adults in this age group) have diabetes. It is estimated that 79.4% live in low- and middle-income countries. Based on the 2019 estimates, by 2030 a projected 578.4 million, and by 2045, 700.2 million adults aged 20-79 years, will be living with

diabetes [https://www.idf.org/e-library/epidemiology-research/diabetes-atlas/159-idf-diabetes-atlas-ninth-edition-2019.html].

According to the IDF 2017, about 425 million people worldwide were estimated to have diabetes and out of them, about 8.8% of people were in the population lives in low and middle-income countries. It is expected that by 2045, about 693 million people between the age group of 18-99 years will be suffering from diabetes [http://diabetesatlas.org/en].

According to a survey done in 2010 in Chipas, United States, it was observed that 38.9% of diabetic people aged around 50 years or older also suffer from DR, out of which, 21.0% suffer from PDR. Diabetes is a major cause of loss of sight and complete blindness in Latin American countries [http://diabetesatlas.org/en].

According to the CDC (Center for Disease Control and Prevention), DR ranks 3rd among all diabetic complications in Mexico. In the region of sub-Saharan Africa, during 2018, total of 2689 patients were studied for DR, out of which 50% were male with an average age of 56 years. Among them, 52% were suffering from DR, 36% of patients have DR which is hazardous for eyes, 7% of T1DM and 5% of T2DM had proliferative DR [Nadarajan *et al*., 2017].

According to the statement by the ADA, (American Diabetes Association) patients who suffered from PDR/NPDR were treated with laser therapy. It minimizes the risk of loss of vision in people with PDR/NPDR. Anti-VEGF drugs given by intravitreal routes are also used and these are more cost-effective than laser therapy. Also, the optimization of glucose level in the blood, rise or fall in blood pressure, and serum lipid levels with the help of scheduled dilated eye examinations can also decrease the risk of loss of vision in the case of DR [Gadkari *et al*., 2016].

The UK became the 1st country to offer systematic screening of DR for all patients diagnosed with diabetes aged 12 years. All patients diagnosed with diabetes are at risk of DR, however, people suffering from T1DM have a higher probability of DR as compared to T2DM. National Diabetic Eye Screening Programme (NDESP), in Wales, it is done by Diabetic Retinopathy Screening Service Wales (DRSSW). In Scotland, screening is run by the Scottish Diabetic Retinopathy Screening (DRS) and

in Northern Ireland by the Diabetic Eye Screening Programme (DESP). Pictures of the dilated pupil were taken by non-mydriatic fundus camera to observe the progression of DR [Zhang *et al*., 2017].

Local therapies used for DR are focal/grid laser therapy, pan-retinal photocoagulation (PRP), and intravitreal anti-VEGF injection. According to the UK Prospective Diabetes Study (UKPDS), metformin helps in reducing the risk of complications related to diabetes when compared with other therapies used for diabetic patients to decrease the blood glucose level [https://health.economictimes.indiatimes.com/news/diagnostics].

In Australia, a DR screening study was carried out for indigenous adults with T2DM in a remote aboriginal community-controlled primary health care clinic in Central Australia and certified non-ophthalmic graders in a retinal grading centre in Melbourne, Australia. Out of 301 participants, 78.7% had DR with an average age group of 48 (19-86) years and had diabetes up to 9.0 years. The prevalence of DR was 47%. sight threatening DR has been observed in 78.0% of detected cases [Bukhari *et al*., 2018].

In Canada, during 2018, higher prevalence rate of DR has been found in the adult population i.e. 40.3%, sight threatening retinopathy is 8.2%. The last prevalence rate of PDR was found to be 23% with T1DM, 14% with T2DM used insulin therapy and 3% used non-insulin antihyperglycemic therapies. The treatments for DR include intraocular injection of pharmacological agents, retinal photocoagulation, and vitreoretinal surgery [Wu *et al*., 2013].

A study conducted from september, 2012 to april, 2013 enrolled 105 T2DM in the district of Tamil Nadu, India . The prevalence of DR in one eye and both the eyes was 32.53% and 31.58% respectively. The severity of DR was moderate (51.9%) followed by mild (44.4%) and severe (3.7%). DR prevalence was high among people with age >60 years with lesser education level. There was no relationship between DR and its duration, treatment regularity, family history of diabetes, hypertension, visual acuity, and cataract [Nadarajan *et al*., 2017].

All India Ophthalmological Society (AIOS) conducted one study on DR from 14th to 21st november 2014. Some diabetic patients were analyzed by team members of the society at 194 centres using a structured protocol provided by society. A total of 6218 diabetic patients were observed, out of which 61.2% were males, 88.6% were in the range of 40 and 80 years of age, almost 2/3rd of the patients belong to west and south zones and had diabetes for more than 5 years. DR prevalence in the entire data set was 21.7% and it was high in males. There were over 72 million cases of diabetes in India during 2017. Diabetes is a major cause of complete blindness and one out of three people suffering from diabetes have DR as well. Therefore, there is an urgent need to raise awareness about DR because it is a major health concern for diabetic people. It is the main cause of loss of vision among adults aged 20-65 years. One of the most common and effective forms of treatment is anti-VEGF therapy, which reduces the leakage from the blood vessels and also reduces the growth of abnormal blood vessels in the eye [Gadkari *et al*., 2014].

Hence, there is an urgent need for the researchers working in the area of DM to understand and rethink the current situation in order to take a step forward for possible treatments for DR.

2.5. Pathophysiology of DR

Diabetes affects millions of people worldwide. It also affects other body parts like kidney, heart, foot, and eyes. Several factors such as hyperglycemia, hyperlipidemia, hypertension, metabolic dysfunction, and ischemia either alone or in combination with other comorbidities can contribute to the development of DR. Increased level of glucose i.e. Hyperglycemia is the main cause of diabetes, which leads to metabolic dysfunction and activation of chronic, low-grade inflammatory signaling which has an important role in DR. These diseases are known to play key roles in the pathogenesis of DR but the exact underlying biochemical mechanisms remain incompletely understood [Fangueiro *et al*., 2015].

In fact, multiple signaling pathways are activated in above-mentioned conditions and are involved in the pathogenesis of DR. The components that get upregulated include polyol pathway, Protein Kinase C (PKC) pathway, oxidative stress, Advanced Glyca-

tion End (AGE) products formation and accumulation, hexosamine pathway, Rennin-Angiotensin-Angiotensinogen System (RAAS), Vascular Endothelial Growth Factors (VEGF), and inflammatory factors [Amadio *et al*., 2010; Lechner *et al*., 2017; Platania *et al*., 2018]. These, in turn, cause molecular, structural, and functional alterations in the diabetic retina (Fig. 2).

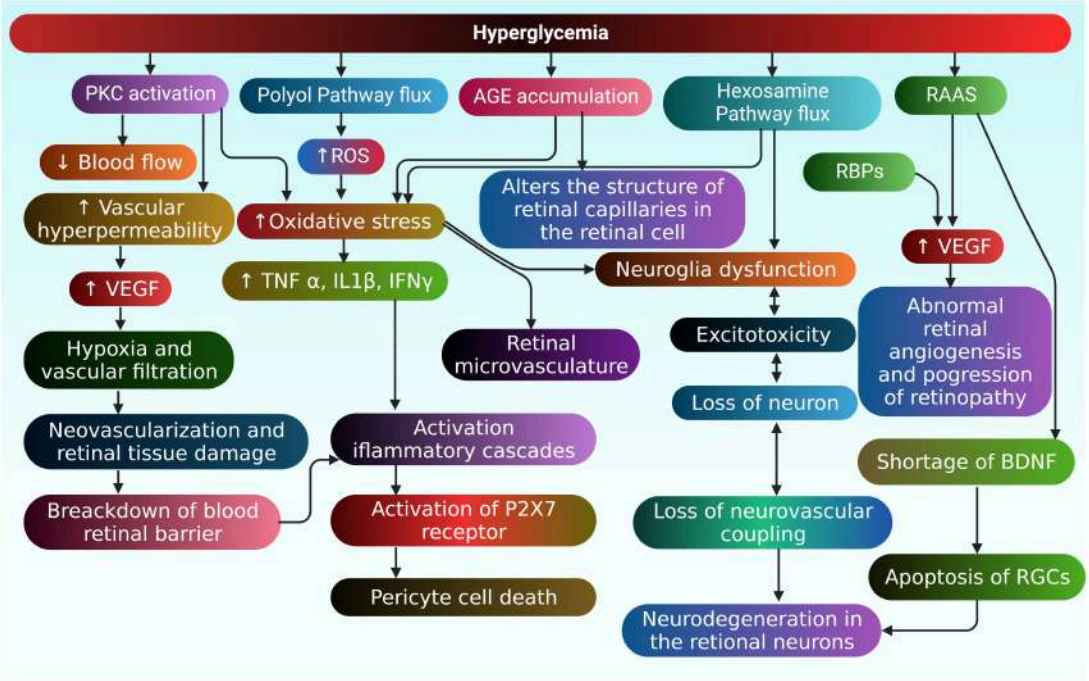


Fig. 2. Pathophysiology of diabetic retinopathy

During the hyperglycemic condition, the polyol pathway is activated by converting glucose into sorbitol in the presence of cofactor nicotinamide adenine dinucleotide phosphate (NADPH), a cofactor. As this cofactor is not available to maintain the activity of glutathione reductase (GR), therefore GR becomes unavailable to maintain the level of reduced glutathione (GSH). Deficiency of GSH, in turn, enhances the levels of reactive oxygen species (ROSp) leading to oxidative stress. This oxidative stress further contributes to structural and functional alterations in the retinal microvasculature [Barot *et al*., 2011; Miwa *et al*., 2003; Naruse *et al*., 2000].

A major manifestation of DR is the microvascular impairment and compromised blood retinal barrier permeability. Increased VEGF levels in response to hypoxia and vascular filtrate may lead to neovascularization and retinal tissue damage in patients

as well as in rodent models of the disease. This breakdown of blood retinal barrier may also aggravate the inflammatory cascades in the DR retina by activation of the P2X7 receptor leading to pericyte cell death [Platania *et al*., 2017; Platania *et al*., 2019]. Human vitreous samples obtained from DR patients indeed revealed enhanced pro-inflammatory IL1β and IFNγ levels along with increased VEGF levels [Tsai *et al*., 2018].

Further studies may be required to establish the time-course of these molecular changes in the retina which in turn may help to develop mechanism-based therapeutics. In addition, activation of the PKC pathway in hyperglycemic condition leads to a decrease in blood flow, an increase in vascular permeability, and eventually retinal neovascularization due to induction of growth factors such as VEGF [Koya *et al*., 1998]. RNA-binding proteins (RBPs) regulate VEGF expression and in DR human antigen R (HuR), RBPs, an expression is upregulated, augmenting in an abnormal increase of VEGF in the diabetic retina [Amadio *et al*., 2016]. Moreover, in hyperglycemic conditions, AGE formation takes place as a by-product due to the reaction of glucose and/or other carbohydrates with proteins, amino acids, nucleic acid, and lipids. AGE accumulation in the basement membranes of retinal cells alters the structure of retinal capillaries [Gardiner *et al*., 2003; Friedman *et al*., 1999]. As the biochemical reactions are altered, for example in the case of deficient glycolysis reactions, there is an excess amount of intracellular glucose and accumulated glucosamine activates the hexosamine pathway. These biochemical alterations are responsible for changes in protein functions, reduce cell protection mechanism, and create an environment of apoptosis in the retinal neurons [Nakamura *et al*., 2001]. Another reason for such alterations is the upregulation of RAAS. RAAS is composed of three major components: renin, angiotensin II, and aldosterone. It increases the VEGF level and leads to abnormal retinal angiogenesis and progression of retinopathy [Franken *et al*, 1998, Luetscher *et al*., 1985]. Thus, an in-depth molecular-level understanding of co tributing factors and biochemical mechanisms will help in a greater understanding of the disease progression and further advances in novel formulation design for the overall management of DR [Fangueiro *et al*., 2015; Elakkiya *et al*., 2017].

There is mounting evidence that suggests DR pathogenesis is related to neurodegeneration of the retina [Seki *et al*., 2004; Liu *et al*., 2013]. Studies have shown that under hyperglycemia conditions retinal ganglion cells (RGCs) undergoes apoptosis due to shortage of neurotrophic factor particularly brain-derived neurotrophic factor (BDNF). Serum of DR patients and animal models showed a significant decrease in the level of BDNF [Ola *et al*., 2012]. Tropomyosin-related kinase B (TrkB) receptor is well known to be expressed in the retina and BDNF has a high affinity for TrkB [Chitranshi *et al*., 2015; Chitranshi *et al*., 2017; Chitranshi *et al*., 2019]. Liu *et al*., have recently demonstrated the neuroprotective role of BDNF through TrkB/ERK/MAP pathway in the hyperglycemic condition in culture retinal neuron cells [Liu *et al*., 2013].

Clinical signs of retinopathy include cotton-wool spots, haemorrhages, lipid exudates, microaneurysms, diabetic macular edema (DME), capillary occlusion, neovascularization. Along with medication, some changes in lifestyle helps in the maintenance of BP and sugar level, other activities like daily exercise, taking a balanced diet along with fresh fruit and vegetables and reduced amount of fat and sugar are helpful in the management of diabetes. Diabetes can be controlled by reducing the abnormalities related to this which will ultimately cure DR too [Sharma *et al*., 2021].

2.6. Current treatment strategies for the management of DR

The treatment or prevention of DR requires synthetic drugs, plant based drugs, laser therapy, and combination therapy. These are discussed below in Table 4.

2.6.1. Role of synthetic drugs

2.6.1.1. Anti-inflammatory drugs

2.6.1.1.1. Corticosteroids

Dexamethasone is a steroid that can be used in the treatment of DR because it will inhibit leukostasis or enhance the barrier function of tight junctions, and also inhibit the release of local inflammatory factors. But dexamethasone has some side effects such as cataract and an increase in intraocular pressure

(IOP). Fluocinolone acetonide is another steroid which can be used in the form of implants for the treatment of DR [Stewart *et al*., 2016].

2.6.1.1.2. Non-steroidal anti-inflammatory drugs (NSAIDS)

NSAIDS such as salicylate drugs (aspirin, sodium salicylate, meloxicam, and sulfasalazine) can be used for the prevention of DR. These drugs protect the diabetic retina from vascular damage and retinal microangiopathy. Aspirin decreases the adhesion of leukocytes to the retina and prevents capillary apoptosis. This work has been reported in streptomycin induced diabetic rat model. Aspirin also has an antiinflammatory effect which helps in the prevention of the DR. Nepafenac act as COX-1 and COX-2 inhibitors and inhibit diabetes-related abnormalities with no side effects on neuron degeneration. Meloxicam is also COX-2 inhibitors which inhibit retinal capillary loss in case of diabetic rats [Kern *et al*., 2001].

2.6.1.2. Antiangiogenic or anti-VEGF agents

Anti-VEGF drugs like ranibizumab, aflibercept, bevacizumab, and pegaptanib play a very important role in DR treatment. They prevent blindness and improve vision in diabetic patients. Bevacizumab is a monoclonal antibody injected intravitreously, it prevents the proliferation of blood vessels in the eye by blocking VEGF factors. Ranibizumab with the same mechanism as that of bevacizumab shows better effect against DR when compared to laser therapy. Aflibercept and Pegaptanib also have a major role in the prevention of DR as anti-VEGF agents. The use of anti-VEGF drugs can cause serious complications of eyes like impaired wound healing, retinal detachment, endophthalmitis, hypertension, proteinuria, and increased risk of CVS diseases [Marashi *et al*., 2016].

2.6.1.3. Vitreous agents

Vitrase (hyaluronidase ovine, ISTA Pharmaceuticals Inc.) is the first and only pure, preservative-free ovine hyaluronidase that is being used for the management of vitreous hemorrhage without any reported serious adverse event in the treated eye [Kuppermann *et al*., 2005]. Intravitreal injections of other pharmacological agents like plasmin and microplasmin are reported to treat diabetic macular edema (DME) and PDR by inducing posterior vitreous detachment and reducing retinal neovascularization. Microplasmin improved the vitreomacular adhesion, cured full thickness macu-

lar hole, and vision impairments in patient reported visual function [Varma *et al*., 2015].

2.6.1.4. Antihyperlipidimics

2.6.1.4.1. Fibrates

These are lipid-lowering drugs used for the treatment of dyslipidemia. Fenofibrate is used for reducing the total cholesterol, low-density lipoproteins (LDL), glycerides, and increase in high density lipoproteins (HDL) levels are due to activation of alpha receptor [Qiu *et al*., 2019].

2.6.1.4.2. Statins

Statins are reported to cause antiangiogenic actions by suppression of VEGF phosphorylation in retinal endothelial cells [Hata *et al*., 2010]. Statins reduce the expression of matrix metalloproteinases (MMP) in retinal pigment epithelial cells. Thus they prevent the breakdown of the BRB [Dorecka *et al*., 2014]. In some of the preclinical studies statin-induced endothelium-dependent and nitric oxide-mediated vasodilation in retinal arteries has also been observed [Nagoaka *et al*., 2007]. In animal models of DR, treatment with statins has been reported to prevent the up-regulation of VEGF and preservation of the BRB through their antioxidant [Fernandes *et al*., 2014; Al-Shabrawey *et al*., 2008; Li *et al*., 2010] and anti-inflammatory effects [Li *et al*., 2009; Miyahara *et al*., 2004]. Somatostatin is one of the first reported drugs that has been used topically as eye drops for the treatment of DR. It can cross the BRB and reach the posterior region of the eye and can show its pharmacological effect by preventing inflammation and vascular leakage [Dal Monte *et al*., 2009]. Simvastatin (20 mg/day) is reported to retard the progression of DR in a randomized clinical trial conducted for 6 months on 50 patients with DM [Bode *et al*., 2018]. In two of the clinical studies, treatment with atorvastatin to 18 patients for 12 months at a dose of 20 mg/day and 30 patients for 4.5 months at a dose of 10 mg/day, led to a reduction in the formation of hard exudates and fluorescein leakage [Panagiotoglou *et al*., 2010; Gupta *et al*., 2004].

2.6.1.5. Antihypertensive agents

2.6.1.5.1. AGE inhibitors

In diabetes, the rate of formation of AGE increases which leads to DR. Aminoguanidine act as (AGE) inhibitors by preventing the accumulation of AGE in precapillary arterioles which decreases the progression of DR. It stops abnormal endothelial cell proliferation and this has been reported in diabetic dogs [Barber *et al*., 2003]. Pyridoxamine is another AGE inhibitor which facilitates the up-regulation of laminin protein. Its mechanism has been reported in diabetic rats in which it decreases AGE level in the diabetic rat retina [Stitt *et al*., 2002].

2.6.1.5.2. RAAS inhibitors

RAAS helps in maintaining BP and fluid balance in the body. The main enzymes are angiotensin-converting enzyme (ACE), rennin, type I and type II angiotensin enzyme which show higher levels of prorenin, renin, and Angiotensin- II (AT-II) in the vitreous humor of patients who suffer from DR. RAAS inhibitors can also decrease the level of retinal VEGF in the patients who suffer from DR. The effect of valsartan was observed in the diabetic rats in which it acts as an AT-II receptor antagonist, which stops the increase in VEGF levels in the retina. Candesartan is an AT-I blocker and its effect was seen in diabetic mice retina. Other inhibitors of AT-I (e.g. losartan and candesartan cilexetil) are undergoing clinical trials for the treatment or prevention of DR. Lisinopril also helps in reducing the progression of DR in diabetic people [Chaturvedi *et al*., 1998].

2.6.1.6. Antiplatelet agents

Platelet activation is one of the side effects of chronic hyperglycemia in addition to retinal-inflammation, aggregation, and thromboxane A2 accumulation. Aspirin is an antiplatelet agent. It is used to slow down the progression of DR when used in combination with dipyridamole [Baudoin *et al*., 1989]. Lisinopril is another antiplatelet agent that helps in reducing the progression of DR in diabetic people [Satofuka *et al*., 2009].

2.6.1.7. Systemic agents

2.6.1.7.1. PKCs inhibitors

PKC regulates angiogenesis, blood flow, and cell permeability to the eye. PKC activity increases because of oxidative stress on vascular endothelial cells which can be inhibited by PKC inhibitors. Pazopanib is a selective inhibitor of glycation that leads to the inhibition of VEGF. Ruboxistaurin (RBX) inhibits the PKC 1 and 2 receptor activity and helps in the prevention of DR. It is a well-tolerated drug and also helps in delaying the time of vision loss [Takahashi *et al*., 2009; Aiello *et al*., 2011].

2.6.1.7.2. Somatostatin

It is one of the first reported drugs which can be used topically as eye drops for the treatment of DR. It can cross the BRB and reach the posterior region of the eye and can show its pharmacological effect by preventing inflammation and vascular leakage [Dal Monte *et al*., 2009].

2.6.1.8. Carbonic anhydrase inhibitors

Carbonic anhydrase is a metalloenzyme that converts carbon dioxide and water to bicarbonate and protons. It plays a significant role in acid-base balance. Retinal pigment epithelium contains membrane-bound carbonic anhydrase. Carbonic anhydrase is present inside the retinal muller cells and red/green cones (not rods). To maintain the pH gradient, carbonic anhydrase is created by the metabolic activity of the cells. An elevated level of carbonic anhydrase was observed in the vitreous of patients with PDR. The carbonic anhydrase inhibitors such as acetazolamide, dorzolamide, benzolamide, on oral administration, downregulate the progression of DR, thus preventing vision loss. Some oral carbonic anhydrase inhibitors such as brinzolamide, methazolamide, ethoxzolamide, butazolamide, dichlorphenamide, and flumethiazide can also be used in the treatment of DR [Henry *et al*., 1996].

2.6.1.9. Growth hormone (GH) inhibitors

The effect of growth hormone (GH) in the pathogenesis of DR was first reported by clinical observation of regression of severe PDR in 1953 [Poulsen *et al*., 1953]. Somatostatin is a growth hormone inhibiting hormone (GHIH) that regulates the endocrine system. Role of several synthetic analogs of somatostatin in the management

of DR has been explored and found to be directly linked with the inhibition of angiogenesis through somatostatin receptors present in endothelial cells [Dal Monte *et al*., 2009]. Octreotide, a somatostatin analog, is being used for the management of DR. The use of octreotide and other somatostatin analogs seems to regulate angiogenic responses to the retinal hypoxic environment through modulation of retinal levels of VEGF and its receptors [Grant *et al*., 2000].

2.6.1.10.Aldose Reductase Inhibitors (ARIs)

In diabetes, the amount of glucose increases and the metabolic pathway is activated to produce sorbitol with the help of aldose reductase enzyme which will lead to an increase increase in oxidative stress. Sorbinil, the 1st ARI which is used for clinical trials showed little effect in control and prevented the development or progression of DR [Naruse *et al*., 2000].

2.6.1.11. Miscellaneous drugs

Some other drugs can also be used for management of DR such as quinolines, tetracyclines such as monocycline, doxycycline, fenofibrate, heparin, 5-Fluorouracil (5-FU). Out of these 5-FU was selected for development of 5-FU loaded NLCs due to its unutilized antiangiogenic activity which can be explored for repositioning of 5-FU for the treatment of DR.

2.6.1.11.1. 5-Fluorouracil (5-FU)

5-FU is anticancer drug having antiangiogenic effect which is found to be effective in DR treatment [Schaub *et al*., 2018]. 5-FU is a BCS class III with poor permeability and bioavailability has been selected in this research work. Hence in the light of aforesaid; a targeted approach such as NLCs were selected to improve 5-FU permeability and bioavailability. Table 3 represents ocular use of 5-FU as per literature.

Table 3: Ocular uses of 5-FU

Title	Conclusion	Reference
A case of sight threatening complications from topical 1% 5-fluorouracil in the treatment of ocular surface squamous neoplasia	Describes serious potential complications of 1% 5-FU, reviews possible risk factors associated with poor outcomes, and discusses our treatment approach	Lin *et al*., 2022
Direct Injection of 5-Fluorouracil Improves Outcomes in Cicatrizing Conjunctival Disorders Secondary to Systemic Disease	Serial injection of 5-FU in the affected fornices is a promising treatment for severe vision-threatening conjunctival scarring from ocular cicatricial pemphigoid and Stevens–Johnson syndrome/toxic epidermal necrolysis	Nina *et al*., 2021
Long-Term Efficacy and Safety of Subconjunctival/Perilesional 5-Fluorouracil Injections for Ocular Surface Squamous Neoplasia (OSSN)	Subconjunctival/perilesional 5-FU injections are an effective and safe treatment for OSSN	Sun *et al*., 2020
Topical 1% 5-FU as a sole treatment of corneo conjunctival ocular surface squamous neoplasia (OSSN)	Topical 5-FU, as a sole therapy, is a long-term safe and effective treatment for patients affected by preinvasive OSSN and for a limited proportion (50%) of invasive OSSN	Zhang *et al*., 2017
Prophylactic intravitreal 5-fluorouracil and heparin to prevent proliferative vitreoretinopathy in high-risk patients with retinal detachment: study protocol for a randomized controlled trial	5-FU is anticancer drug having antiangiogenic effect which is found to be effective in DR treatment	Schaub *et al*., 2018
Formulation and In vitro Evaluation of Nanoparticulate Drug Delivery System Loaded With 5-FU	The formulated nanoparticulate delivery system of 5-FU was capable of exhibiting sustained release action for a period of 24 h	Gavini *et al*., 2014

2.6.2. Plant-based agents

Drugs from plant origin are also used for the treatment of diabetes and its complications including DR from very old time. The herbal medicines are beneficial in DR as they are considered safe, with no or less side effects, decrease elevated blood glucose level and provide a protective effect on the retina. Some natural aldose reductase inhibitors (ARIs), derived from plant sources are *Ganoderma lucidum, Tinospora cordifolia,* and *Ocimum sanctum*. *Ganoderma lucidum* protects the retina from oxidative damage [Wang *et al*., 2005]. *Tinospora cordifolia* exhibits its protective action by preventing retinal oxidative stress (ROS) produced by proangiogenic overexpression and proinflammatory mediators [Agrawal *et al*., 2012]. *Ocimum sanctum* exerts its protective effect against DR when used in combination with vitamin E [Halim *et al*., 2006]. Curcumin, isolated from *Curcuma longa*, helps in treating DR by inhibiting

VEGF and thus having anti-VEGF action [Aldebasi *et al*., 2013]. It also shows antioxidant and anti-inflammatory effects in the experimental rat retina [Gupta *et al*., 2011].

In one of the studies, the effects of curcumin on human retinal pigment epithelial (RPE) cells exposed to high glucose (HG) insult were performed on RPE cells that were cultured both in normal and HG conditions to assess the effects of curcumin on the cell viability, nuclear factor erythroid 2-related factor 2 (Nrf2) expression, HO-1 activity, and extracellular-signal-regulated kinase (ERK) 1/2 expression. RPE cells exposed to HG insult were treated with curcumin. The obtained results indicated that treatment with curcumin caused a significant decrease in terms of apoptosis. It was also able to induce HO-1 expression via Nrf2 activation and counteracted the damage elicited by HG. Hence it was concluded that curcumin provided protection against HG induced damage in RPE cells through the activation of Nrf2/ HO-1 signaling that involved the ERK pathway [Bucolo *et al*., 2019].

In another study, self-assembling aqueous ocular nanomicellar eye drop loaded with curcumin was formulated using quality by design approach to treat age-related macular degeneration (AMD) in the eye. The results indicated that curcumin loaded nanoformulation exhibited significant protection of retinal (D407) cells against H_2O_2-induced oxidative stress. In addition to that, the study conducted using ELISA (Enzyme-Linked Immunoassay) indicated a significant reduction in VEGF release in D407 cell lines by this formulation, which is an indication of a reduction in risk of angiogenesis. *In vitro* drug release kinetics suggested a sustained drug release profile indicating a long-term protection ability of the developed formulation against oxidative stress to retinal cells [Alshamrani *et al*., 2019].

Pycnogenol, extracted from the bark of the maritime pine tree demonstrated its protective effect by providing antioxidant and anti-inflammatory activity in DR [Steigerwalt *et al*., 2009]. Genistein was found to be effective in the prevention of retinal vascular leakage in a rat model [Nakajima *et al*., 2001]. Green tea also reduces the progress of DR due to its antioxidant properties [Kumar *et al*., 2012]. Hesperetin, quercetin, and rosmarinic acid have also exhibited a reduction in DR by preventing

angiogenesis via antioxidant activity [Parveen *et al*., 2018]. The plant-based dietary supplements such as flavonoids, carotenoids, vitamin A, C (ascorbic acid), and E (tocopherols) possess strong antioxidant effect and prevent lipid peroxidation in the body system. Resveratrol has shown protection against oxidative stress in retinal pigment epithelial cells [Neal *et al*., 2020]. The use of α-tocopherol helps in the prevention of diabetes-induced abnormal retinal blood flow [Fangueiro *et al*., 2015].

2.6.3. Combination Therapies

Some of the combination therapies (as depicted in Fig. 3 below) are also available for the treatment of DR, which shows better results as compared to monotherapy (synthetic drugs or plant based drugs) [Gillies *et al*., 2011; Paccola *et al*., 2008; Shimura *et al*., 2008; Huang *et al*., 2009; Cho *et al*., 2009; https://clinicaltrials.gov/ct2/show/NCT02432547; Brown *et al*., 2006].

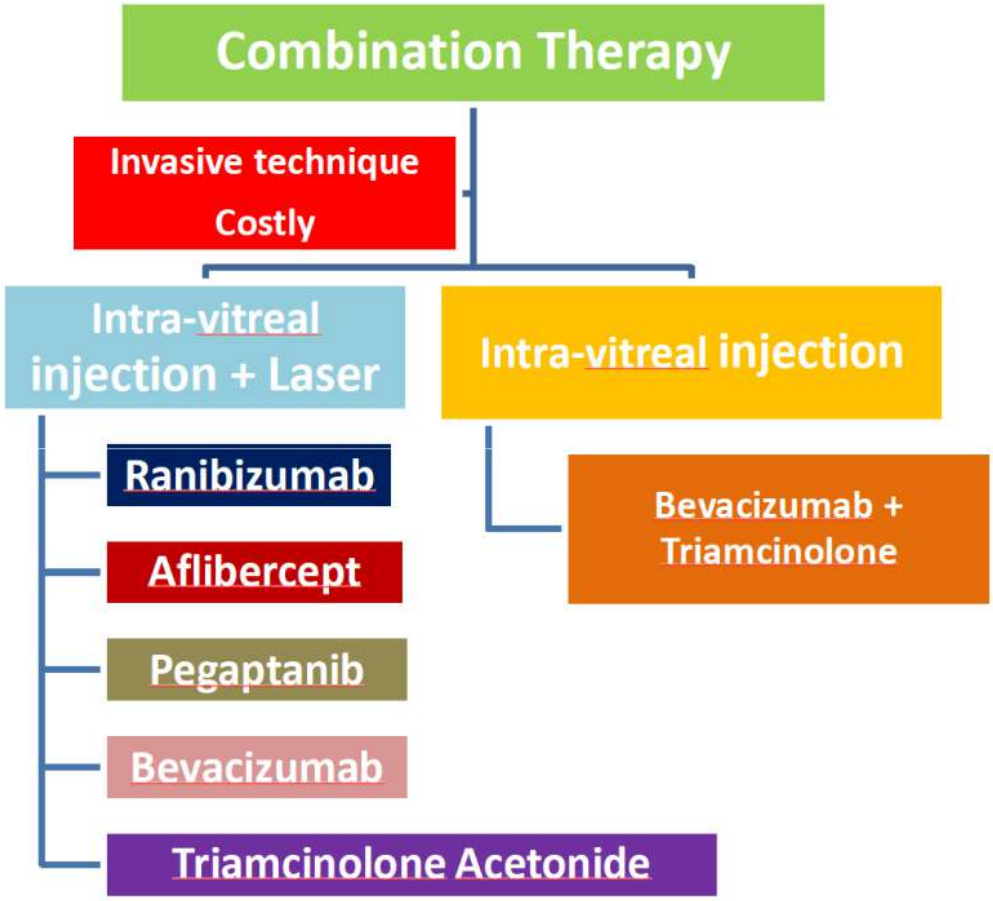


Fig. 3. Combination therapies available for the treatment of diabetic retinopathy

Table 4: Drugs explored to manage DR

Category	Mechanism of action	Example of drugs	Marketed formulations	Dosage form	Adverse effects	Reference
Anti-Inflammatory Steroids (Corticosteroids)	Anti-inflammatory and anti-angiogenesis activities by modulating various proinflammatory mediators, such as tumor necrosis factor-alpha (TNF-α), interleukin-1β (IL-1β), and VEGF	1.Triamcinolone acetonide	1. Triesence® (Alcon pharmaceuticals, Geneva, Switzerland) 2. Trivaris® (Allergen, Irvine, CA, USA) 3. Kenalog® 40, (Bristol-Myers-Squibb, Princeton, NJ)	Intravitreal Injection	Cataract, glaucoma and elevation of intraocular pressure Retinal detachment, vitreous hemorrhage, and endophthalmitis	Danis *et al.*, 2000
		2. Sustained-release triamcinolone acetonide	I-vation® (Surmodics, Inc., USA)	Biodegradable intravitreal implant	Elevation of intraocular pressure	Lu *et al.*, 2018
		3. Sustained release fluocinolone acetonide	1. Iluvien® (Alimera Sciences Inc., Alpharetta, GA, USA) 2. Retisert® (Bausch & Lomb Inc., Rochester, New York, USA)	Non-biodegradable intravitreal insert	Cataract, glaucoma and elevation of intraocular pressure	Campochiaro *et al.*, 2011
		4. Sustained release Dexamethasone	1. Ozurdex (Allergen, Irvine, CA, USA) 2. Posurdex®, (Allergen, Irvine, CA, USA)	Biodegradable intravitreal implant	Elevation of intraocular pressure	Lu *et al.*, 2018

		5. Tissue-activated proprietary corticosteroid prodrug	Cortiject implant (NOVA63035; Novagali Pharma)	Intravitreal implant in the form of emulsion	Not reported	
		6. VerisomeTM liquid delivery system based sustained release triamcinolone acetonide	IBI-20089 (Icon Bioscience Inc., Newark, Calif.)	Intravitreal injection		
NSAIDs	COX-1 and COX-2 inhibitors	Nepafenac	Nevanac® (Alcon pharmaceuticals, Geneva, Switzerland)	Topical	Not reported	Elakkiya *et al.*, 2017
	COX-2 inhibitor	Meloxicam	Mobic® (OEM manufacturers In dia)	Oral	Blurred vision, conjunctivitis, optic neuritis	
	Anti-inflammatory by modulating TNF-α	Etanercept	Enbrel® (Amgen Inc., and Wyeth, Germany)	Intravitreal injection	Not reported	
	Anti-inflammatory by modulating TNF-α	Infliximab	Remicade® (Janssen Biotech, Inc., Philadelphia, USA)	Intravitreal injection		
Antiangiogenic agents	Primary targets the subfamily protein, VEGF, in which its over-expression plays a crucial role in the progression of DR and AMD	1. Bevacizumab	Avastin® (Genentech, South San Francisco, CA)	Intravitreal injection	Retinal detachment, Endophthalmitis, Impaired wound healing, Hypertension, Proteinuria	Schmidinger *et al.*, 2011
		2. Ranibizumab	Lucentis® (Genentech, South San Francisco, CA)	Intravitreal injection	Retinal detachment, Endophthalmitis, Impaired wound healing, Hypertension, Proteinuria	Matsumiya *et al.*, 2011

		3. Aflibercept	EYLEA® (Regeneron Pharmaceuticals Inc., and Bayer HealthCare Pharmaceuticals, New York, USA)	Intravitreal injection	Retinal detachment, Endophthalmitis, Impaired wound healing, Hypertension, Proteinuria	Moradi *et al.*, 2013
		4. Pegaptanib	Macugen® (Bausch and Lomb, USA)	Intravitreal injection	Retinal detachment, Endophthalmitis, Impaired wound healing, Hypertension, Proteinuria	Gonzalez *et al.*, 2009
		5. Integrin alpha5beta1 antagonist	JSM6427 (under clinical trial, Jerini AG, Germany)	Intraocular implanted osmotic pump that releases drug for six months	Not reported	
		6. Ubiquitin-like-conjugating enzyme	ATG3 (under clinical trial by CoMentis Inc., USA)	Topical eye drop		
		7. Pazopanib	Votrient® (Glaxo SmithKline, USA)	Oral		Takahashi *et al.*, 2009.
Vitreous Agents	Reported to treat DME and PDR by inducing posterior vitreous detachment	1. Vitrase	Hyaluronidase ovine (ISTA Pharmaceuticals Inc., Irvine, CA)	Intravitreal injection	Retinal detachment, Endophthalmitis, Impaired wound healing, Hypertension, Proteinuria	Kuppermann *et al.*, 2005

		2. Microplasmin	Ocriplasmin® (ThromboGenics NV, Belgium)	Intravitreal injection	Retinal detachment, Endophthalmitis, Impaired wound healing, Hypertension, Proteinuria	Lopez-Lopez *et al.*, 2009
Systemic agents (Hypolipidemic Agents)	1. Fibrates They act via non-lipidemic mechanisms, mainly activating PPAR-α for DR prevention. Activation of PPAR-α mediated by fenofibrate led to inhibition of VEGFR2 expression and neovascularization in human umbilical endothelial cells	Fenofibrate	Tricor® (AbbVie) Lipofen (Kowa Pharmaceuticals America Inc) Lofibra® (Teva), Lipanthyl, Lipidil, Lipantil micro and Supralip (Abbott Laboratories) Fenocor-67 (Ordain Health Care) Fibractiv 105/35 (Cogentrix Pharma, India), Fenogal (SMB Laboratories) Antara (Oscient Pharmaceuticals) Stanlip (Ranbaxy, India)	Oral	Blood clot, Headache, back pain, Nausea	Chen *et al.*, 2007

	2. Statins These are used to treat hyperlipidemia by mainly lowering the triglyceride levels, total and low-density lipoprotein (LDL) cholesterol, small LDL cholesterol particles, and apolipoprotein B while increasing high-density lipoprotein (HDL) cholesterol.	1. Simvastatin 2. Rosuvastatin	Zocor® (Merck & Co., Inc. USA)	Oral	First pass metabolism, Less therapeutic effect	Dal Monte *et al.*, 2009
Systemic agents (Antihypertensive Agents)	ACE-2 inhibitors	1. Enalapril Maleate	Vasotec® (BTA Pharmaceuticals, Inc. USA)	Oral	Intestinal angioedema,	Mauer *et al.*, 2009
		2. Lisinopril		Intravitreal injection	Retinal detachment, Endophthalmitis, Impaired wound healing, Hypertension, Proteinuria	Satofuka *et al.*, 2009
	RAAS inhibitors [Angiotensin receptor blocker (ARB)]	1. Losartan	Cozaar® (Merck & Co., Inc. USA)	Oral	1-2% drug reaches to the retina of eye. Repeated dose required. Less therapeutic effect	Mauer *et al.*, 2009
		2. Candesartan	Atacand® (Astra Zeneca, Europe)	Oral	Hypotension, Reduction in GFR, Hyperkalemia, Anaemia	Chaturvedi *et al.*, 2008

Antiplatelet agents	Reduction in microaneurysms formation	Dipyridamole + Aspirin	Aggrenox® (Boehringer Ingelheim, Germany)	Oral	Hearing problem, yellowing of eye, (Jaundice), Liver problem	Group *et al.*, 1989
Systemic agents	Treats moderate to severe NPDR by reducing the overactivation of protein kinase C beta, which is involved in the pathogenesis of DR	Ruboxistaurin	Arxxant® (Eli Lilly, Carolina, USA)	Oral	First pass effect, Less therapeutic effect	Aiello *et al.*, 2011
Systemic agents	Somatostatin Derivatives (rates of progression to PDR, vitreous haemorrhage, and the need for vitrectomy in patients with severe NPDR)	Octreotide	Sandostatin® (Novartis, Switzerland)	Topical	Increase of IOP, cataract and endophthalmitis	Grant *et al.*, 2005
Systemic agents (Carbonic anhydrase inhibitors)	Inhibition of activity of carbonic anhydrase	1. Acetazolamide 2. Dorzolamide 3. Benzolamide 4. Brinzolamide 5. Methazolamide 6. Ethoxzolamide 7. Butazolamide 8.Dichlorphenamide 9. Flumethiazide	Trusopt® (Dorzolamide) (MSD pharmaceuticals, India), Azopt® (Benzolamide, Alcon pharmaceuticals, Geneva, Switzerland)	Oral	Temporary blurred vision, redness of the eye, red eye	Henry *et al.*, 1996

2.7. Limitations of current therapies

The major reasons for the moderate success of existing therapies include protein binding, reduction in drug's concentration on instillation, less space at ocular site, invasive process, and cost of treatment [Selvaraj *et al*., 2017].

- **Protein binding-**Drug binding with proteins present in tear fluid leads to a decrease in the absorption of the drug.
- **Reduction in drug's concentration**-There is a decrease in the drug concentration on administration due to various defence mechanisms including tear formation, blinking, and flow of the material via nasolacrimal duct. Metabolism and enzymolysis of the drugs also occur due to the presence of enzymes in the eye.
- **Less space**-Limited capacity of the conjunctival sac, which is approximately 30 μL without blinking. This often leads to spillage of the drug upon administration.
- **Anatomical barriers**-Complex anatomical structure of the eye, little transparency of the cornea, low absorptive surface, and lipophilicity of corneal epithelium, and BRB are the major reasons for the limited success of conventional dosages forms in several ocular diseases when administered through ocular route.
- **Expensive**-The intravitreal injections are expensive. For example, Ranibizumab (Lucentis) costs approximately 2,150 USD per dose, pegaptanib sodium costs 1,000 USD per dose, bevacizumab (Avastin) 55 USD per dose and aflibercept (Eylea) costs €943 per injection [Gonzalez-Cordero *et al*., 2013].

The challenges of the afore mentioned existing conventional dosage forms suggest to rethink on the need for non-invasive, effective and economical delivery systems. In recent years NDDS have emerged as an effective carrier that can deliver the drug effectively to retina overcoming the challenges associated with conventional as well as surgical approaches. The two main approaches in improving the ocular bioavailability are by prolonging the contact time on ocular surface and increasing the corneal permeability. The main aim of NDDS is to reduce the cost and frequency of injections, increase therapeutic effect, decreases the side effects, improved patient compliance, and overcome the limitations associated with the conventional dosage forms [Sharma *et al*., 2021].

2.7.1. Blood-Retinal Barrier (BRB)

BRB does not allow the movement of drugs from blood into the posterior part of the eye. The retinal pigment epithelium cells (RPE) and retinal capillary endothelial cells (RCE) are made up of the outer and inner BRBs, respectively. The latter is the peculiar cells present between the choroid and neural retina, which assist the visual system by uptake of retinoids and selective molecular transport between photoreceptors and chorio capillaries. On the contrary, the tight junctions in these cells impede the intercellular permeation.

The outer BRBs, limit the drug entry from the choroid into the retina. Although, a systemic administration of drugs is the ideal route for delivery into the retina but BRB stringently modulates drug penetration into retina from the blood. Therefore, for the transportation of drugs from the choroid into the retina, specific/targeted drug delivery systems or different routes of drug administration, for example, suprachoroidal, systemic, periocular, sub-retinal, or topical are entailed.

Current advancements at nanoscale level have invigorated formulation scientists to overcome BRB. Different nanoparticles, for example, solid lipid nanoparticles (SLNs), polymeric nanoparticles, nanostructured lipid carriers (NLCs), gold nanoparticles (GNPs), liquid crystals (LC), liposomes and microemulsions have several advantages compared to conventional drug delivery systems, such as increased surface area, improved adhesion, depot formation, enhanced biocompatibility and controlled drug release rate. In addition, they reduce the side effects of the drugs and increase patient compliance. Different advancements in the field of nanotechnology to specifically surpass the BRB have been discussed below.

Kim and co-workers investigated GNPs, to cross the BRB, in two different sizes i.e. 100 nm and 20 nm and administered them intravenously. They demonstrated that GNPs with a size of 20 nm were uniformly distributed throughout the retina (endothelial cells, neurons, peri-endothelial glial cells) without any cytotoxicity. The authors hypothesized that the size, shape, and chemical composition provided such distribution patterns [Kim *et al*., 2009].

Furthermore, the concept of gene delivery has also been explored by the researchers to target retina. The study aimed to transport a non-viral plasmid explicated with gene targeting technology by non-invasive intravenous administration. The pegylated immune liposomes (size 85 nm) encapsulated an expression plasmid, promoter glial fibrillary acidic protein (GFAP) gene, simian virus (SV) 40 and were functionalized with rat 8D3 monoclonal antibody (mAb) in order to target transferrin receptor (TfR) rich structures. The results revealed pervasive exogenous gene expression throughout the retina because of the modulation by cell-specific promoter and elimination of gene expression in peripheral tissues by tissue-specific promoter. Another study reported non-viral retinal gene delivery using arginine-glycine-aspartic acid (RGD) peptide, nano-engineered transferrin, or dual-functionalized poly-(lactide-co-glycolide) nanoparticles administered through intravenous route for effective management of choroidal neovascularization (CNV). In the case of functionalized nanoparticles, intraceptor gene expression in retinal vascular endothelial cells, photoreceptor outer segments, and retinal pigment epithelial cells could be obtained as compared to non-funtionalized nanoparticles. A well-established model i.e. Laser-induced CNV model was used. The reason for retinal targeting could be attributed to the leaky BRB created by CNV due to laser treatment in rat eye [Singh *et al*., 2009]. Campbell and coworkers examined RNAi-mediated systemic delivery to the retina. The results demonstrated reversible modulation of the inner BRB in mice by repression of the transcript, which encodes a protein component i.e. claudin, that forms the paracellular pores and channels for selective ion permeability of the inner retinal vasculature [Campbell *et al*., 2009].

2.8. Topical drug delivery system for posterior eye segment

The topical route (Fig. 4 below) is the most common route for delivery of the drug to the eyes because of its high patient compliance and non-invasive technique. Mostly, the drug is not able to cross the barriers and is unable to reach the posterior eye segment. Nano-drug delivery system aids in better permeability of the drug across the cornea, conjunctiva, and sclera by topical route [Koevary *et al*., 2003].

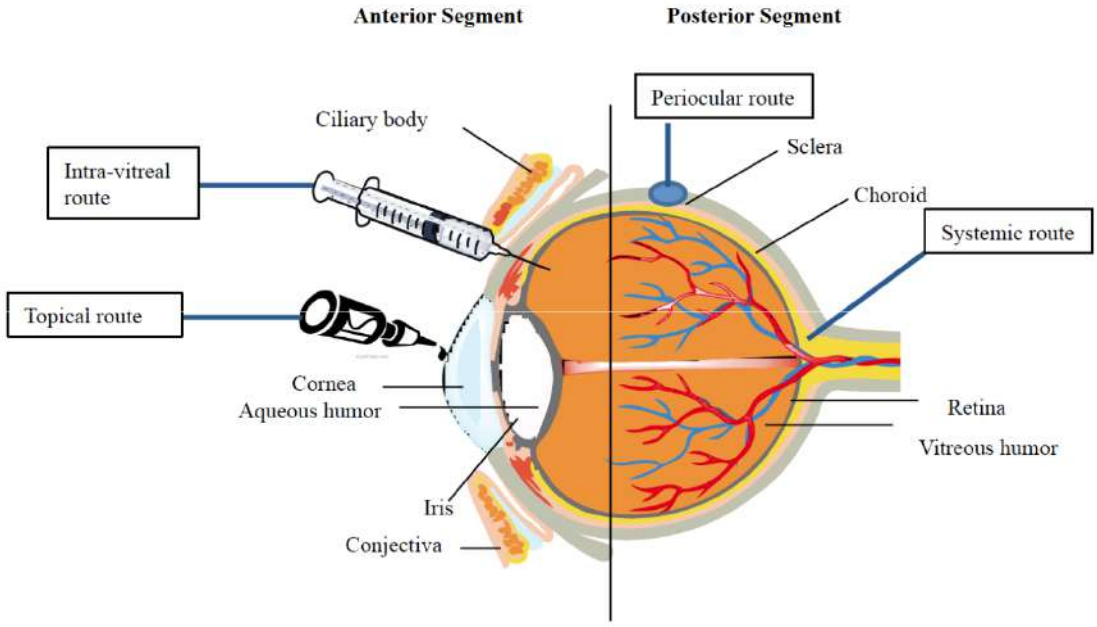


Fig. 4. Different routes and anatomy of eye

The next section describes the different drugs applied topically for efficient delivery into the posterior segment of the eye. Dexamethasone, a steroid, was formulated with cyclodextrin (water-soluble) microparticles in a form of suspension. The drug was successfully delivered to the posterior part of the eye. In another study, the micellar formulation of dexamethasone and cyclosporin were successfully able to achieve therapeutic concentrations and reach the targeted retinal layers [Sigurdsson *et al*., 2007].

Furthermore, the topical application of NSAIDs such as nepafenac decreased VEGF production and prevented choroidal and neovascularization. The study demonstrated that even large molecular weight drugs could not only penetrate the posterior part of the eye but can also be therapeutic. A carbonic anhydrase inhibitor, dorzolamide hydrochloride, was also administered for eye as eye drops and was found to be rapidly distributed in ocular tissues [Inoue *et al*., 2004].

Furrer *et al*., investigated the difference between systemic and topical administration (eye drops) of a tumor necrosis factor (TNF)-alpha inhibitory single-chain antibody fragment, (scFv) ESBA105 for local drug distribution in a rabbit's eye. The researchers observed a significantly higher concentration of ESBA105 in all the ocular

compartments after topical administration. Thus, based on the observed results, the authors proposed ESBA105 as a promising molecule to be delivered through the topical route for the treatment of ophthalmological disorders. Similarly, these two different routes were compared to other routes such as intravenous and intranasal to determine effective dexamethasone concentration. The drug absorption after topical route dominated in the anterior eye segment. A maximum amount of the drug (60%) was released into retina when administered through the topical route. Betaxolol, an anti-glaucoma drug when applied topically, was found to be localized in the posterior eye segment due to enhanced permeability across cornea and conjunctiva [Furrer *et al*., 2009].

2.9. Potential of nanocarriers in ocular drug delivery

The delivery of drugs through the evolving nanocarriers offers promising alternative to overcome the associated challenges with current therapies such as anti-VEGF administration. NDDS in terms of nano carriers such as liposomes, NPs, SLNs, NLCs, lipid and polymeric nanoparticles, cationic nanoemulsions, microspheres, prodrugs, dendrimers, polymeric gels, and ocular inserts etc., are non-invasive and applied through ocular route for their possible targeting to retina which offers site specific release of drug thereby dose reduction, enhanced drug bioavailability, improved permeation of drug to the retinal areas, enhancement of residence time, better ocular tolerability etc.

The main aim of development of NLCs is to reduce the cost and frequency of injections, increase therapeutic effect, decreases the side effects, improved patient compliance, and overcome the limitations associated with the conventional dosage forms. Topical route is the most common route for delivery of the drug to the eyes because of its high patient compliance and non-invasive technique. Mostly the drug is not able to cross the barriers and unable to reach the posterior eye segment. Nanocarriers aids in better permeability of drug across the cornea, conjunctiva, and sclera by topical route [Gaudana *et al*., 2010]. Table 5 having the different examples of nanocarriers used for ocular drug delivery.

Table 5: Different nanocarriers used for ocular drug delivery

Novel drug delivery system	Preparation method	Drug	Route of administration	Remarks	References
Liposomes	• Thin lipid film hydration • Extrusion method	Citicoline	Topical administration	• Prevention of glial activation and neural apoptosis in diabetic retina	Bogdanov *et al.*, 2018
	• Film hydration method	Timolol maleate chitosan coated liposomes	Topical administration	• Enhancement of ocular permeation, precorneal residence time, and bioavailability	Tan *et al*., 2017
	• Rotary evaporation method	Ranibizumab	Subconjunctival injection to retina	• Higher drug concentration achieved in the retina	Joseph *et al*., 2017
Nanoparticles (NPs)	• Rotary evaporation method	PEG-PLA chains modified with a cell penetrating peptide (CPP)	Intravenous injection	• Reduces neovascular lesion size • Enhanced drug accumulation	Wang *et al*., 2019
	• Extensive analysis	Magnetic nanoparticles	Intraocular delivery	• Enhanced drug bioavailability	Amato *et al*., 2018
	• Emulsification evaporation method	Connexin43 mimetic peptide	Intravitreal injection	• Reduces possible ocular complications • Preventing the loss of the photoreceptor	Nor *et al*., 2018

Solid Lipid nanocarriers (SLN)	• Hot homogenization and ultrasonication method	Triamcinolone acetonide	Topical administration	• Drug delivery platform for deeper ocular tissues	Tatke *et al*., 2018
	• Cold homogenization method	Myricitrin	Oral	• Antioxidant • Antiapoptotic • Antidiabetic	Ahangarpour *et al.*, 2018
	• Ultrasonic melt-emulsification method	Ciprofloxacin	Intravenous injection	• Controlled release and a superior antibacterial effect	Shazly *et al*., 2017
Nanostructured lipid carriers (NLC)	• High pressure homogenization	Itraconazole	Topical	• Antiangiogenic activity • Improved retention and ocular permeability	Selvaraj *et al*., 2019
	• Cold homogenization	Palmitoylethanolamide (PEA)	Topical	• Improved corneal permeability • High ophthalmic tolerability • Prolonged retention capacity • Mucoadhesive and film forming properties	Paterniti *et al*., 2015

2.10. Nanostructured lipid carriers (NLCs) in DR

NLCs are drug delivery systems that are generally prepared with both types of lipids, i.e., solids as well as liquids. NLCs can be used topically which can reduce the pain as well as discomfort related to intravitreal injections. Various advantages of NLCs are discussed below:

1. Enhancement of solubility of the hydrophobic drugs in different dosage forms.
2. Ability to enhance the storage stability of different dosage forms.
3. Improvement in permeability and bioavailability of different drugs.
4. Reduction in the adverse effects of some drugs.
5. Prolonged biological half-life of drugs.
6. Targeted delivery of the drug to different tissues in the body can be achieved.
7. Sustained release of drugs achieved in the form of coated NLCs.

Because of the above-mentioned advantages, nowadays NLCs are getting more attention.

In one of the studies, researchers repositioned itraconazole (ITR) NLCs to manage DR owing to its potent unutilized antiangiogenic. High-pressure homogenization method was used to prepare ITR-NLCs (Fig. 5(a)). Tripalimitin (liquid lipid), Capmul MCM (solid lipid), Tween 80 (surfactant), and Transcutol HP (cosurfactant) were used to prepare ITR-NLCs. Further, surface modification of optimized NLCs was done using chitosan to modify the surface charge and to improve the retention and ocular permeability of drug. An antiangiogenesis study was carried out using the HET-CAM (Hen's Egg Test-Chorioallantoic Membrane) model, which indicated the excellent antiangiogenic potential of ITR-NLCs (Fig. 5(b)). These NLCs also exhibited anti-neovascularization effect on the rat cornea (Fig. 5(c)) [Selvaraj *et al*., 2019].

In another study, palmitoylethanolamide (PEA) was found to show beneficial effects in several retinal diseases such as DR, glaucoma, etc. PEA attenuated the degree of retinal inflammation while preserving the blood-retinal barrier in diabetic rats [Paterniti *et al*., 2015].

Platania *et al*., (2019) developed Myriocin (Myr) loaded NLCs for the treatment of retinitis pigmentosa (RP). The formulation was prepared using melt emulsification

and ultrasonication technique using tween-80 as a surfactant and Gelucire 44/14 (10% w/v) and Mygliol 812 (5% w/v) as lipids. The ocular distribution of the Myr-NLC formulation in rabbits and C57BL6J mice was carried out and performance was compared with Myr-aqueous suspension and Myr-loaded SLNs. Myr-NLC formulation provided significantly ($p<0001$) higher myriocin retinal availability, after topical ocular administration, compared to myriocin suspension or Myr-SLN formulation [Platania *et al*., 2019].

The delivery of ocular drugs through the evolving nanocarriers such as NLCs offers promising alternative to overcome the associated challenges with current therapies. The NLCs provide enhanced drug bioavailability, improved permeation of drug to the retinal areas, enhancement of residence time, non-invasive delivery, better ocular tolerability etc. Despite these advantages, patient compliance as well as safety always remain a prime concern. Further more research is going on in the area of gene therapy, ocular implant, stem cell therapy as well as laser therapy for the treatment of posterior segment of eye. These advancements offer great benefits in providing the drug in a safer, effective and more complaint way. In addition to that generation of stable and scalable nanocarrier is always a challenge for the pharmaceutical scientist and industries. Moreover, the availability of these advancements for the clinical use require utmost efforts of interdisciplinary research collaboration.

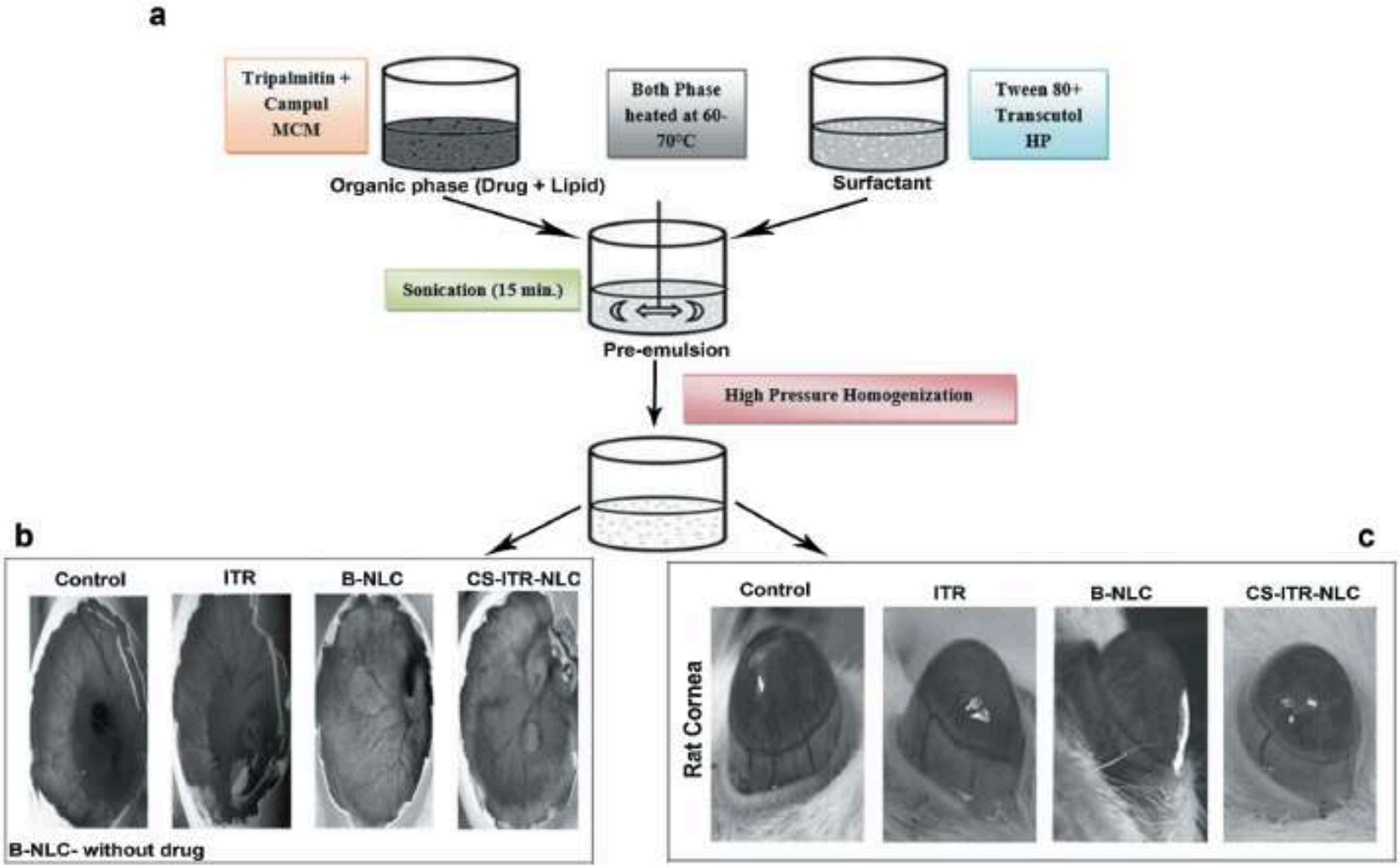


Fig. 5. Preparation and characterization of NLCs (a) Formulation of NLCs (b) Antiangiogenesis study by HET CAM model (*ex vivo* chorallantoic membrane assay) (c) Rat corneal images for antineovascularization Copyright @ 2019, Mary Ann Libert, Inc., publisher

2.11. Surface modification

NLCs are promising drug delivery system having more drug loading, controlled release, more bioavailability [Beloqui *et al*., 2013]. However, as per reported literature anionic nature of NLCs difficult to interact with the negatively charged corneal surface. To overcome this problem surface modification of NLCs can be done to convert this negative charge to positive charge with cationic polymers such as eudragit-RS-100, chitosan etc., [Khursheed *et al*., 2022; Luo *et al*., 2011; Selvaraj *et al*., 2019].

For improvement of corneal penetration and precorneal retention time, surface modification of developed genistein (GEN)-loaded NLCs with cationic eudragit-RS-100 was there for interaction of NLCs with corneal cells which results in improved penetration and longer retention [Zhang *et al*., 2014].

Chitosan (CS) coated nanoparticles have been found to be more effective in terms of the amount of the drug permeated through the cornea compared to uncoated particles [Liu *et al*., 2016]. CS food-grade polysaccharide polymer consisting of 2-amino-2-deoxy-h-d-glucan having glycosidic linkages with positive charge, which is recognized as a good candidate for modification of NLCs [Khursheed *et al*., 2022]. It is having the ability to adhere to mucosal surface owing to its positive charge leading to prolonged residence time at drug absorption sites, biocompatibility, biodegradability, non-toxicity, mucoadhesiveness and non-allergenic nature [Kean *et al*., 2010; Khursheed *et al*., 2022; Nagarwal *et al*., 2009]. Drug bioavailability can improve through improvement of residence time and penetration enhancement across mucosal barriers [Khursheed *et al*., 2022; Gan *et al*., 2013]. Despite the versatility of nanoparticulate drug delivery systems coated with CS, only those with an optimal release rate are suitable for ophthalmic use. CS having the ability to open tight junctions of epithelium and increasing the paracellular transport of drug molecules [Du *et al*., 2011].

3. HYPOTHESIS OF RESEARCH

As mentioned in the introduction and review of literature section that there are several treatment options available for the management of DR such as injections, laser therapy, implants, etc. for targeting the drug to the retina of the eye. However, these therapies are expensive and compromised patient compliance.

The major reasons for the moderate success of existing therapies include protein binding, reduction in drug's concentration on instillation, less space at ocular site, invasive process, and high cost of treatment. The challenges of the aforementioned existing conventional dosage forms suggest to rethink on the need for non-invasive, patient compliance, and cost effective systems. In recent years NDDS have emerged as an effective carrier that can deliver the drug effectively to retina overcoming the challenges associated with conventional as well as surgical approaches. In recent years several nanotechnology based drug delivery systems (called as nanomedicines) have been explored to prevent diabetes related complications.

DR is a multi factorial process which involves inflammation and ischemia. After the retinopathy, early and regular treatment must be given to the patient to prevent the vision loss. The best medication for the DR's treatment should be highly therapeutic, safe, and have longer duration of action. Hence, it is important to develop a NDDS based formulation containing drug that could effectively treat DR at an optimum dose and minimum side effects. In addition, it should be able to protect the drug from off targets and make them to reach at retina.

Different nanoparticles, for example, solid lipid nanoparticles, polymeric nanoparticles, nanostructured lipid carriers, gold nanoparticles, liquid crystals, liposomes, and microemulsions have several advantages as compared to conventional drug delivery systems, such as increased surface area, improved adhesion, depot formation, and controlled drug release rate. In addition, they reduce the side effects of the drugs and increase patient compliance, overcoming the challenges associated with treatments with drugs alone or, even with classic delivery systems such as need of high doses to

exhibit therapeutic effect, high toxicity, reduced half-life, aggregation, enzymatic, and chemical degradation.

Because of the above-mentioned advantages, nowadays NLCs are getting more attention. In one of the studies, researchers repositioned itraconazole (ITR) NLCs to manage DR owing to its potent unutilized antiangiogenic effect [Selvaraj *et al*., 2019]. Similarly, 5-FU is also having the antifibrotic, anticancer, and anti-angiogenic effect similar to anti-VEGF drugs. Selection of 5-FU due to it potent unutilized anti-angiogenic activity for the management of DR will be explored. Hence, NLCs were selected as a delivery system and surface modification of 5-FU-NLCs can also be done using chitosan (CS) to improve the bioadhesion. CS have the ability to adhere to mucosal surface leading to prolonged residence time at drug absorption sites, biocompatibility, biodegradability, non-toxicity, mucoadhesiveness and non-allergenic nature. CS-5-FU-NLCs can tightly adhere to the retina of the eye because of the presence of lipid molecules and transport the drug in a more controlled fashion for the treatment and management of DR as shown in Fig. 6.

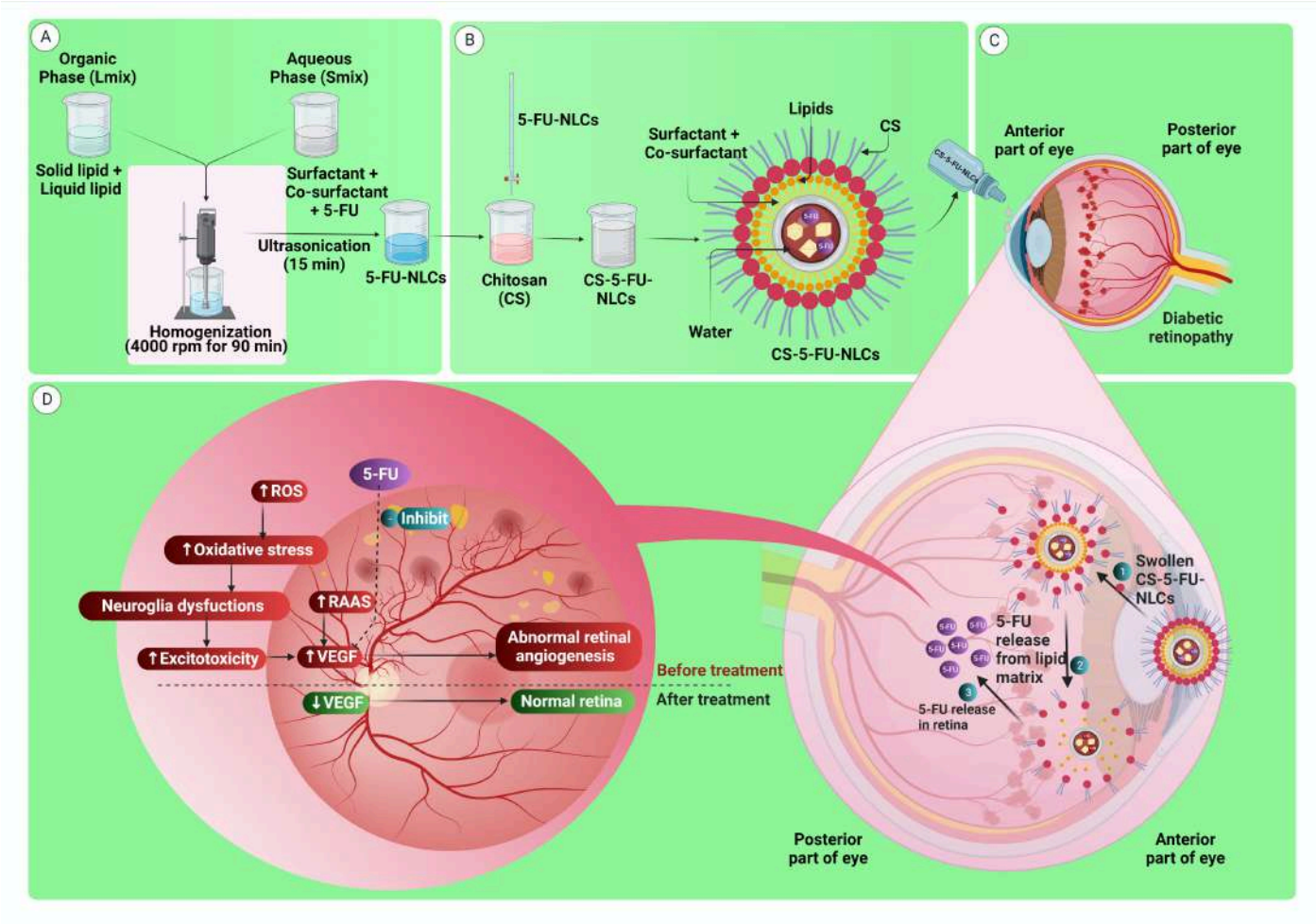


Fig 6. Hypothesis of research work

4. AIM OF RESEARCH PROPOSAL

Design, development and characterization of ocular Nanostructured Lipid Carriers (NLCs) of 5-FU for the treatment of DR.

4.1. OBJECTIVES OF THE RESEARCH PROPOSAL

The major objectives of the present investigation are to:

1. Formulate and optimize the NLCs of 5-FU using quality by design approach
2. Improve the drug retention time and permeability of the formulation by surface modification using chitosan
3. Administer the formulation via ocular route and enhancing its penetration to the retina
4. Study the safety of developed surface modified 5-FU-NLCs using Hen's Egg Test-Chorioallantoic Membrane (HET-CAM) assay
5. Evaluate the antineovascularization efficacy of surface modified 5-FU-NLCs in streptozotocin induced rats with DR

5.1. MATERIALS AND METHOD

5.1.1. Materials

The list of various materials used are summarized in Table 6.

Table 6: List of materials used in study

Chemicals	Manufacturers
Acetic acid	Loba Chemie Pvt. Ltd. Mumbai, India
Almond oil	Dee-Jay Corporation, Jalandhar, India
Castor oil	Central Drug House Pvt. Ltd., New Delhi, India
Campul MCM	M/S Abitec Corp., Ohio
Cotton seed oil	Dee-Jay Corporation, Jalandhar, India
Chitosan	Dee-Jay Corporation, Jalandhar, India
Calcium chloride	Dee-Jay Corporation, Jalandhar, India
Dialysis Membrane	Dee-Jay Corporation, Jalandhar, India
Distilled water	Lovely Professional University, Jalandhar, India
Ethanol	Central Drug House Pvt. Ltd., New Delhi, India
Eucalyptus oil	Central Drug House Pvt. Ltd., New Delhi, India
5-Fluorouracil	Loba Chemie Pvt. Ltd., Mumbai, India
Glyceryl monostearate	Dee-Jay Corporation, Jalandhar, India
Groundnut oil	Dee-Jay Corporation, Jalandhar, India
Hematoxylin and eosin	Dee-Jay Corporation, Jalandhar, India
Labrasol ALF	Gattefosse Pvt Ltd., Mumbai, India
Labrasol	Gattefosse Pvt Ltd., Mumbai, India
Labrafac Lipophile WL	Gattefosse Pvt Ltd., Mumbai, India
Labrafil M 2125 CS,	Gattefosse Pvt Ltd., Mumbai, India
Mustard oil	Dee-Jay Corporation, Jalandhar, India
Methanol	Dee-Jay Corporation, Jalandhar, India

n-octanol	Central Drug House Pvt. Ltd., New Delhi, India
O-phosphoric acid	Dee-Jay Corporation, Jalandhar, India
Olive oil	Dee-Jay Corporation, Jalandhar, India
PG 200	Loba Chemie Pvt. Ltd., Mumbai, India
PG 400	Loba Chemie Pvt. Ltd., Mumbai, India
PG 600	Loba Chemie Pvt. Ltd., Mumbai, India
PEG 200	Loba Chemie Pvt. Ltd., Mumbai, India
PEG 400	Loba Chemie Pvt. Ltd., Mumbai, India
PEG 600	Loba Chemie Pvt. Ltd., Mumbai, India
Potassium chloride	Dee-Jay Corporation, Jalandhar, India
Potassium phosphate mono basic	Dee-Jay Corporation, Jalandhar, India
Paraformaldehyde	Dee-Jay Corporation, Jalandhar, India
Paraffin wax	Dee-Jay Corporation, Jalandhar, India
Transcutol HP	Loba Chemie Pvt. Ltd., Mumbai, India
Tween 20	Loba Chemie Pvt. Ltd., Mumbai, India
Tween 80	Loba Chemie Pvt. Ltd., Mumbai, India
Soyabean oil	Loba Chemie Pvt. Ltd., Mumbai, India
Sodium phosphate dibasic	Dee-Jay Corporation, Jalandhar, India
Sodium bicarbonate	Dee-Jay Corporation, Jalandhar, India
Sodium chloride	Dee-Jay Corporation, Jalandhar, India
Sesame oil	Loba Chemie Pvt. Ltd., Mumbai, India
Span 80	Loba Chemie Pvt. Ltd., Mumbai, India
Sodium pyruvate	Loba Chemie Pvt. Ltd., Mumbai, India
Streptozotocin	Anjan Enterprises, Amritsar, India

5.1.2. Equipment and Instruments

The list of various equipment and instruments used are summarized in Table 7.

Table 7: List of equipment and instruments used in study

Equipment	Model/Manufacturers
Centrifuge	REMI RM-12C, Remi Electrotechnic Ltd, Varsi, Mumbai, India.
Differential scanning calorimeter	DSC 6000 Perkin Elmer, USA DSC Q200 V24.4 Build 116, Bangalore, India
Electronic weighing balance	CY 360, Shimadzu Co. Ltd., Kyoto, Japan Sansui-vibra DJ-150S-S, India
Fourier transform infrared spectroscopy	8400S, Perkin Elmer, IndiaMeditech Technologies India Pvt. Ltd
Franz diffusion cell	EMFDC-07, Meditech Technologies India Pvt. Ltd, Mumbai, India.
Fundus camera	UTAS-3000 ERG system, LKC Technologies, Mumbai, India.
High performance liquid chromatography	HPLC LC-20AD, Shimadzu Co. Ltd., Kyoto, Japan
Hot air oven	Cadmach Drying Oven, Cadmach Machinery Ltd., Ahmadabad, India
Homogenizer	Glass-Teflon potter homogenizer, Thomas Scientific, USA
Magnetic stirrer	Remi 5MLH, Vasai, Mumbai, India
Microscope camera	Leica CTR 5000, Leica microsystems, Germany
Melting point apparatus	Popular, India
Particle size analyzer	Malvern Zetasizer, Nano ZS90, UK
pH meter	Phan, Lab India, Mumbai, India
Powder x-ray diffractometer	Bruken D8 Advance, USA
Scanning electron microscope	Joel JSM-7610F Plus, Japan
Shaking water bath	Labfit, India
Stability Chamber	REMI CMH 10S, Remi Sales & Engineering Ltd, Mumbai, India
Transmission electron microscope	JEM-2100 plus Electron microscope, Jeol, Japan

Ultrasonication bath	UC-8120, Loba Life, Lobachemie, Mumbai, India
UV Spectrophotometer	UV-1800, Shimadzu Co. Ltd., Kyoto, Japan
Vortex mixer	REMI CM101, Delhi, India.
0.22 mm syringe filter	Hi Media, India

5.2. Methodology

5.2.1. Analytical method development

High Performance Liquid Chromatography (HPLC, HPLC LC-20AD, Shimadzu Co. Ltd., Kyoto, Japan) is an instrument which can be used to identify the drug, determine the quantity of drug, and isolation of different components present in mixtures. The HPLC system consists of delivery pump for mobile phase (LC- 20 AD; Shimadzu, Japan), a detector (PDA-photodiode array) (SPDM20A; Shimadzu, Japan), a 20 µL loop (Rheodyne), and software LC Solution [Garg *et al*., 2018]. A Nucleodur C18 column (Reverse phase, 250 mm × 4.6 mm, particle having 5 micron size) used as stationary phase and combination of OPA (Ortho-phosphoric acid, 0.5%) and methanol ratio 95:5 v/v used as mobile phase in an isocratic elution mode [Sharma *et al*., 2020b]. The solvent sample mixture (5-FU solution) and mobile phase passes through a HPLC column (stationary phase) and then into a detector, where an electronic output is given as a chromatograph signal [Mujeeb *et al*., 2014].

5.2.2. Estimation of 5-FU by RP-HPLC

Different composition of mobile phase solvents system being used, out of which one reliable ratio was selected for short run time and good resolution for 5-FU estimation. The waste was collected in a vessel outside the machine. The flow rate of mobile phase was 0.8 mL/min and 5-FU solution volume of the injection was 20 µL. The eluent was observed at 266 nm. The blank (without 5-FU) was also injected under the same conditions and HPLC was run for 20 min [Jyoti *et al*., 2019]. The chromatograph signals area i.e. mean peak area was calculated for repeated samples of the same drug solution [Sharma *et al*., 2020b]. For validation of this developed method different parameters were estimated such as selectivity, sensitivity, precision (inter-day and intra-day), system suitability, accuracy, and linearity according to International Conference on Harmonization guidelines i.e. ICH Q2 (R1) [https://www.gmp-compliance.org/guidelines/gmp-guideline/ich-q2r1-validation-of-analytical-procedures-text-and-methodology].

5.2.2.1. Stock solution preparation

Stock solution of 5-FU was prepared by accurately weighing 5-FU (10 mg) on digital weighing balance (CY 360, Shimadzu Co. Ltd., Kyoto, Japan) and dissolving it in 2 mL of distilled water in 10 mL volumetric flask using vortex mixer (REMI CM101, Delhi, India). Then make up the volume up to 10 mL with distilled water to prepare 1 mg/mL stock solution. Sonicate the stock solution for 10 min. to completely dissolve the 5-FU [Sharma *et al*., 2020b].

Then, transferred 1 mL of this solution into volumetric flask (100 mL) and diluted up to 100 mL with distilled water. Sonicated this solution for 10 minutes to form 10 µg/mL concentration. Similarly, other concentrations were prepared such as 2, 4, 6, and 8 µg/mL from the stock solution in different volumetric flasks [Ali *et al*., 2007].

5.2.2.2. Development of calibration curve

From the above prepared standard 5-FU stock solution 2, 4, 6, 8, and 10 mL was transferred into different flasks (10 mL) with the help of pipette and make up the volume up to 10 mL with purified water. Sonicated all the samples for 10 min. Peak area of all the samples was determined by using HPLC method. Six injections were given for each concentration. The peak of the 5-FU was observed at 266 nm and the average peak area was calculated from all six peaks of different concentrations. The calibration curve (CC) was plotted between concentrations of 5-FU between 2-10 µg/mL concentration (x-axis) versus the area of the peak (y-axis). The regression coefficient was determined [Garg *et al*., 2018]. Different parameters for validation of the developed HPLC method was performed.

5.2.3. Validation of developed HPLC method

Validation was done for the developed method by determining system suitability, linearity, accuracy, precision, LOD, LOQ, precision (inter-day and intra-day), and specificity study as per ICH Q2 (R1) guidelines.

5.2.3.1. System suitability

To check system suitability, injections (n=6) of standard 5-FU solutions (6 µg/mL) was injected to HPLC. The theoretical plates number, peak asymmetry, HETP i.e.

height equivalent to theoretical plate and retention time (RT) were also measured [Jin *et al*., 2005].

5.2.3.2. Linearity and Range

To determine the linearity and range, calibration curve was plotted between 5-FU concentrations from 2-10 µg/mL (x-axis) versus area of the peak (y-axis). The regression coefficient was calculated which represents linearity of the curve [Abdulrahman *et al*., 2011]. The range was given between the upper and lower limit interval of 5-FU concentration. This calibration curve was used further to determine other parameters for HPLC developed method validation [Mohanta *et al*., 2018].

5.2.3.3. Accuracy

The accuracy of an analytical method describes the closeness of result between the value which is accepted either as a conventional true value or an accepted reference value and the value found. Accuracy of the experiment was established by using recovery studies from the selected concentration range of calibration curve from 2-10 µg/mL, mid concentration of the drug (6.0 µg/mL) was taken as MQC [100%]. Similarly, LQC [80%] and HQC [120%] of 6.0 µg/mL were also prepared from the stock solution. Suitable aliquots of 4.8, 6.0, and 7.2 mL were withdrawn from stock solution and transferred individually into different flasks (10 mL) and make up the volume up to 10 mL to prepare LQC, MQC and HQC respectively.

Six injections were given repeatedly and the mean area of the observed peaks were calculated for all six injections. For the determination of accuracy of this method mean percentage recovery of the drug was calculated from all these three concentrations. % absolute recovery was calculated by dividing the actual recovery of drug to their theoretical concentration and multiplying them by hundred (Equation 1) [Mittal *et al*., 2000].

$$Absolute\ percent\ recovery = \frac{Actual\ concentration\ recovered}{Theoretical\ concentration} \times 100$$

….Equation 1

5.2.3.4. Precision

The precision of an analytical method defined the closeness of results between measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. For determining precision of this method % RSD was calculated for the six observations of LQC, MQC, and HQC solutions at interday (all three dilutions made in three different days), intraday (all three dilutions made within same day) and interanalyst (all three dilutions made by three different analysts) with same experimental conditions [Sharma *et al*., 2020b].

5.2.3.5. Estimation of LOD and LOQ

The LOD i.e. Limit of Detection of an analytical method is defined as the lowest concentration of sample or drug which can be detected**.** The LOQ i.e. Limit of Quantification of an analytical method is defined as the lowest concentration of sample or drug which can be determined quantitatively**.** Both were calculated to determine sensitivity of this method by using standard deviation of response (σ) and slope of standard curve (S). Standard deviation of Y intercepts of regression line was used as standard deviation [Awad *et al*., 2018]. Equation 2 and Equation 3 for LOD and LOQ, respectively as follow:

$$LOD = 3.3\frac{\sigma}{s} \quad \text{.....Equation 2}$$

$$LOQ = 10\frac{\sigma}{s} \quad \text{.....Equation 3}$$

5.2.4. Preformulation studies of 5-FU

5.2.4.1. Physical description of 5-FU

The physical appearance of 5-FU was examined by taking 1 g sample on watch glass and observes its different organoleptic properties such as color, odour, state, and presence of any foreign matter [https://www.drugbank.ca/drugs/DB00544**]**.

5.2.4.2. Melting point determination of 5-FU

Meting point of 5-FU was determined by two methods-Capillary fusion (Popular, India) method and Differential Scanning Calorimetry **(**DSC Q200 V24.4 Build 116, Bangalore, India).

5.2.4.2.1. Capillary fusion method

This method was used to determine melting point of the 5-FU using melting point apparatus (Popular, India). 5-FU was filled in the capillary tube, sealed at one end and kept in the melting point apparatus such that the sealed end is down towards the apparatus. The temperature at which the drug starts to melt was noted down with the help of thermometer and compared with literature value [https://pubchem.ncbi.nlm.nih.gov/compound/3385#section=Chemical-and-Physical-Properties].

5.2.4.2.2. Differential scanning calorimetry (DSC)

DSC is a thermo analytical technique in which the difference between the amount of heat required to increase the temperature of a sample and the reference is measured as a function of temperature and time. It is used extensively to determine the melting point, purity, glass transition temperature and thermal decomposition of drug. 5-FU (2.5 mg) was heated in a pierced aluminium pan from 0 to 350 ºC at a heating rate of 10 ºC/min under a steam of nitrogen at a flow rate of 50 mL/min. The empty aluminium pan was taken as reference [Tummala *et al*., 2015].

5.2.4.3. Fourier Transform Infrared Spectroscopy (FTIR)

In order to find characteristic functional group of 5-FU, FTIR spectroscopy was carried out by FTIR (Perkin Elmer, India Meditech Technologies India Pvt. Ltd, 8400S). The sample (5 mg) was taken and mixed with KBr in the ration of 1:3 and pressed using hydraulic press. A thin disc was prepared and subjected for spectral analysis. Scan was taken at wavelength 4000 cm^{-1} to 400 cm^{-1} [Pharmacopoeia, I. 2018**].**

5.2.4.4. X-Ray diffraction (XRD)

The XRD spectra of pure drug was recored using X-ray diffractometer (Bruken D8 Advance, USA) using copper line as the radiation source. X-ray beams were incident on the sample at a current of 40-mA and voltage of 40-kV. The scanning rate of sample was kept at 0.010^{o} min^{-1} over a 2θ range of 5-80º [Kumar *et al*., 2018].

5.2.4.5. Partition coefficient (PC) determination

For determination of PC or log P of 5-FU, 25 mL water and 25 mL n-octanol was kept overnight for saturation in a separating funnel. 25 mg of 5-FU was added to it and kept for 24 h with intermittent shaking. After separation of organic and aqueous layer filter each layer separately and estimated the concentration of drug in both the layers using HPLC (n=3).The PC (Log P) was calculated using Equation 4 [Mutalik *et al*., 2014].

$$Log\ P = \frac{Conc\ of\ drug\ in\ organic\ layer}{Conc\ of\ drug\ in\ aqueous\ layer} \quad \text{.....Equation 4}$$

5.2.4.6. Solubility studies of 5-FU

The solubility of 5-FU in the lipids was the major concern that influences the drug entrapment efficacy (EE) and *in vitro* drug release. 5-FU solubility was performed in selected solvents such as STF buffer, distilled water, OPA buffer, PBS buffer, ethanol, methanol and 10% ethanol, various surfactants and cosurfactants (Transcutol HP, Tween 20 and 80, PG 200, 400 and 600, PEG 200, 400 and 600, Span 80), solid lipids and liquid lipids (Glyceryl monostearate (GMS), Labrafil M 2125 CS (LMCS), Labrafac Lipophile WL, Labrasol ALF, Groundnut oil, Soyabean oil, Olive oil, Cotton seed oil, Mustard oil, Almond oil, Eucalyptus oil, Castor oil, Sesame oil). In centrifuge tube (CT), 1 mL of each solid lipid, liquid lipid, surfactants, and cosurfactants were taken separately. An excess amount of 5-FU (50 mg) was added to each glass vial and mixed using vortex mixer. It was kept over water bath shaker for 24 h at 37/100 ºC. After this, centrifugation of this mixture was done at 5000 g for 5 min. The supernatant was filtered through membrane filter (0.22 µm). The percentage solubility of 5-FU (Equation 5) was determined by injecting the samples to HPLC (n=3) and record their areas [Sharma *et al*., 2020b; Kumar *et al*., 2020a].

$$Percentage\ solubility = \frac{Practical\ Concentration}{Theoretical\ Concentration} \times 100 \text{.....Equation 5}$$

5.2.5. Construction of Ternary Phase Diagram (TPD)

The results of solubility studies have shown high solubility of 5-FU in solid lipid (GMS), liquid lipid (LMCS), surfactant (Tween 80), and cosurfactant (Transcutol HP). These excipients were selected further to prepare NLCs by modified melt emulsification and ultrasonication method as per Fig. 7 [Malik *et al*., 2018]. By ultrasonication method, ultrasonic waves (20 kHz) provide cavitation forces to break droplets of coarse emulsion into nanoemulsion [Taha *et al*., 2020]. Organic phase (2% w/v) was prepared by mixing L_{mix} in which different ratios of solid lipid: liquid lipid (1:2, 1:1, 2:1) were placed in a beaker on magnetic stirrer above melting point of solid lipid (70 ºC). Aqueous phase (1% w/v) was prepared by mixing S_{mix} in different ratios of surfactant: cosurfactant (1:2, 1:1, 2:1) in 50 mL of deionized water in a beaker at 70 ºC. This would lead to decrease in interfacial tension at higher temperature there is adsorption of surfactant at oil-water interface [Bergfreund *et al*., 2021], which would result in formation of stable NLCs of smaller particle size and prevention of droplet's coalescence. Further, the L_{mix} and S_{mix} were varied from 1:9 to 9:1 ratio [Khursheed *et al*., 2022]. Using these ratios, a total 81 formulations were prepared (N1 to N81) as per composition shown in Table 8. Molten organic phase (L_{mix}) was added dropwise (1 mL/min) to the aqueous phase (S_{mix}) with stirring at 500 rpm on magnetic stirrer to form microemulsion. After this, 50 mL of ice-cold water was added to the microemulsion with constant stirring to form NLCs. Finally, homogenize the later formulation i.e. NLCs for 90 min at 4000 rpm and sonicated for 15 min on ultrasonic water bath. The NLCs were stored for 24 h to check their stability. The study was designed using Triplot software version 4.1.2. (Todd Thompson Software) and ternary phase diagram (TPD) was created. The regions indicating the formation of clear and transparent NLCs formation with absence of precipitation or phase separation were selected as NLCs. Other NLCs were rejected due to translucent appearance [Khursheed *et al*., 2022]. The results of TPD shown that 81 prototypes prepared, N14 having L_{mix} ratio 1:1 and S_{mix} 1:2 with internal ratios of solid lipid: liquid lipid (2.5:2.5) and surfactant:cosurfactant (1.7:3.3) shown minimum PS, higher ZP, and narrow PDI which are important parameters for good NLCs (refer result and discussion section 6.3).

Hence, this formulation (N14) was selected for further development of 5-FU-NLCs [Sharma *et al*., 2018, Ghosh *et al*., 2020].

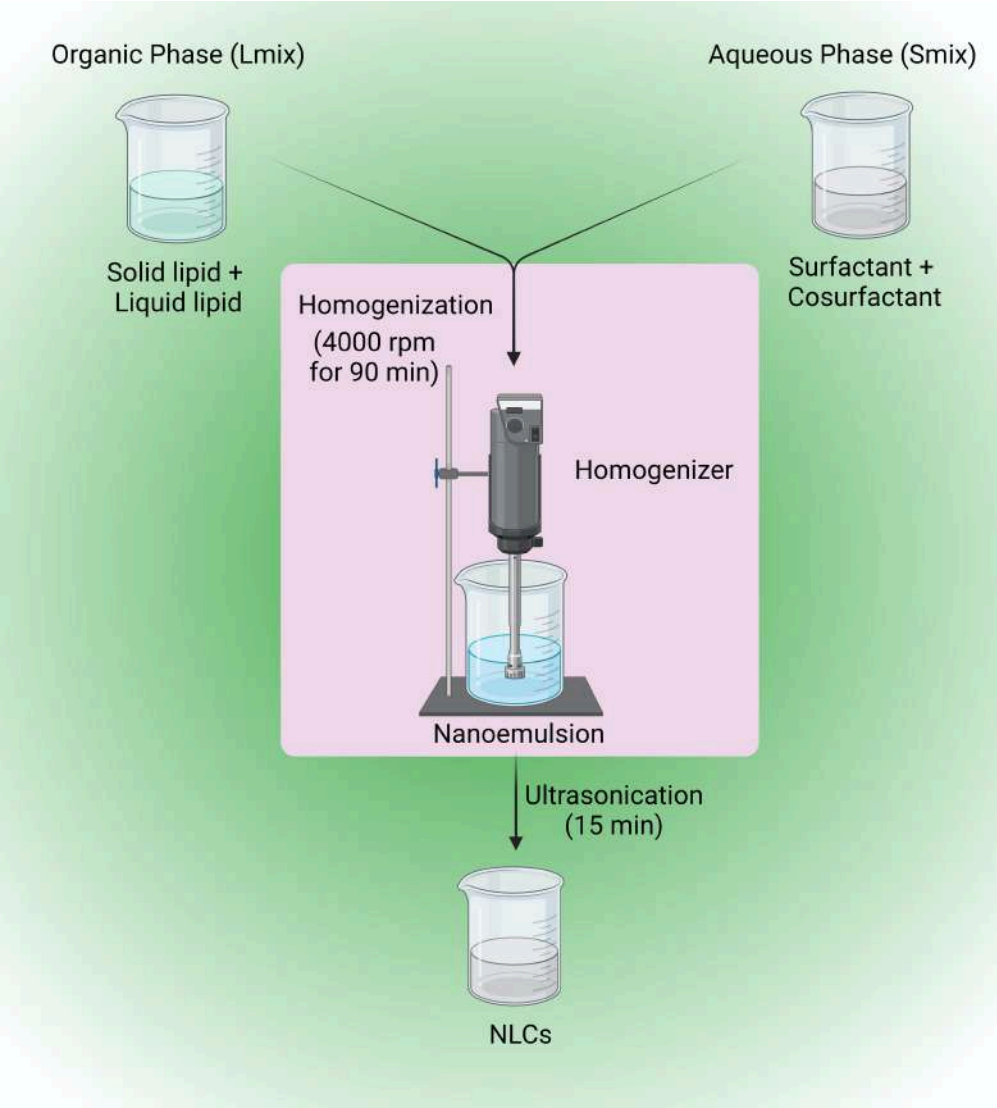


Fig. 7. Preparation of NLCs by melt emulsification method

Table 8: Composition of N1 to N81 formulation as per TPD

Formulation code	L_{mix} GMS:LMCS (1:1)	S_{mix} T80:Transcutol (1:1)	Formulation code	L_{mix} GMS:LMCS (1:2)	S_{mix} T80:Transcutol (1:1)	Formulation code	L_{mix} GMS:LMCS (2:1)	S_{mix} T80:Transcutol (1:1)
N1	0.5:0.5	4.5:4.5	**N28**	0.3:0.7	4.5:4.5	**N55**	0.7:0.3	4.5:4.5
N2	1.0:1.0	4.0:4.0	**N29**	0.7:1.3	4.0:4.0	**N56**	1.3:0.7	4.0:4.0
N3	1.5:1.5	3.5:3.5	**N30**	1.0:2.0	3.5:3.5	**N57**	2.0:1.0	3.5:3.5
N4	2.0:2.0	3.0:3.0	**N31**	1.3:2.7	3.0:3.0	**N58**	2.7:1.3	3.0:3.0
N5	2.5:2.5	2.5:2.5	**N32**	1.7:3.3	2.5:2.5	**N59**	3.3:1.7	2.5:2.5
N6	3.0:3.0	2.0:2.0	**N33**	2.0:4.0	2.0:2.0	**N60**	4.0:2.0	2.0:2.0
N7	3.5:3.5	1.5:1.5	**N34**	2.3:4.7	1.5:1.5	**N61**	4.7:2.3	1.5:1.5
N8	4.0:4.0	1.0:1.0	**N35**	2.7:5.3	1.0:1.0	**N62**	5.3:2.7	1.0:1.0
N9	4.5:4.5	0.5:0.5	**N36**	3.0:6.0	0.5:0.5	**N63**	6.0:3.0	0.5:0.5
		S_{mix} T80:Transcutol (1:2)			**S_{mix} T80:Transcutol (1:2)**			**S_{mix} T80:Transcutol (1:2)**
N10	0.5:0.5	3.0:6.0	**N37**	0.3:0.7	3.0:6.0	**N64**	0.7:0.3	3.0:6.0
N11	1.0:1.0	2.7:5.3	**N38**	0.7:1.3	2.7:5.3	**N65**	1.3:0.7	2.7:5.3
N12	1.5:1.5	2.3:4.7	**N39**	1.0:2.0	2.3:4.7	**N66**	2.0:1.0	2.3:4.7
N13	2.0:2.0	2.0:4.0	**N40**	1.3:2.7	2.0:4.0	**N67**	2.7:1.3	2.0:4.0
N14	2.5:2.5	1.7:3.3	**N41**	1.7:3.3	1.7:3.3	**N68**	3.3:1.7	1.7:3.3

N15	3.0:3.0	1.3:2.7	**N42**	2.0:4.0	1.3:2.7	**N69**	4.0:2.0	1.3:2.7
N16	3.5:3.5	1.0:2.0	**N43**	2.3:4.7	1.0:2.0	**N70**	4.7:2.3	1.0:2.0
N17	4.0:4.0	0.7:1.3	**N44**	2.7:5.3	0.7:1.3	**N71**	5.3:2.7	0.7:1.3
N18	4.5:4.5	0.3:0.7	**N45**	3.0:6.0	0.3:0.7	**N72**	6.0:3.0	0.3:0.7
		S_{mix} T80:Transcutol (2:1)			**S_{mix} T80:Transcutol (2:1)**			**S_{mix} T80:Transcutol (2:1)**
N19	0.5:0.5	6.0:3.0	**N46**	0.3:0.7	6.0:3.0	**N73**	0.7:0.3	6.0:3.0
N20	1.0:1.0	5.3:2.7	**N47**	0.7:1.3	5.3:2.7	**N74**	1.3:0.7	5.3:2.7
N21	1.5:1.5	4.7:2.3	**N48**	1.0:2.0	4.7:2.3	**N75**	2.0:1.0	4.7:2.3
N22	2.0:2.0	4.0:2.0	**N49**	1.3:2.7	4.0:2.0	**N76**	2.7:1.3	4.0:2.0
N23	2.5:2.5	3.3:1.7	**N50**	1.7:3.3	3.3:1.7	**N77**	3.3:1.7	3.3:1.7
N24	3.0:3.0	2.7:1.3	**N51**	2.0:4.0	2.7:1.3	**N78**	4.0:2.0	2.7:1.3
N25	3.5:3.5	2.0:1.0	**N52**	2.3:4.7	2.0:1.0	**N79**	4.7:2.3	2.0:1.0
N26	4.0:4.0	1.3:0.7	**N53**	2.7:5.3	1.3:0.7	**N80**	5.3:2.7	1.3:0.7
N27	4.5:4.5	0.7:0.3	**N54**	3.0:6.0	0.7:0.3	**N81**	6.0:3.0	0.7:0.3

5.2.6. Screening and optimization of formulation variables

Design of Experiment **(**DoE) plays an important role in optimization of various product and process parameters and have direct impact on product quality [Khursheed *et al*., 2022]. Box Behnken design (BBD) is one of the most commonly used response surface models as compared to other study designs such as Doehelrt and central composite designs, as BBD possess advantages like requirement of less experimental points (three levels per factor) and high efficiency [Mohanta *et al*., 2018].

The developed formulation i.e. N14 (as per section 5.2.5) was optimized by BBD for 4 factors at 3 levels using Design expert software (Design Expert, Version 11.0.1, Stat-Ease Inc., Minneapolis, MN) [Singare *et al*., 2010]. Four factors such as solid lipid concentration (A, mg), liquid lipid concentration (B, µL), surfactant concentration (C, µL) and cosurfactant concentration (D, µL) as independent variables and they were set at low (-1), medium (0), and high (+1) levels on the basis of initial trials results (Table 9). As per the experimental design, total 29 experimental runs were performed and accordingly, 29 prototypes (F1 to F29) were developed along with 5-FU , The 5-FU (2 % w/v) was dissolved in aqueous phase (S_{mix}) as per section 5.2.5. and characterized for PS as Y1 and ZP as Y2, PDI as Y3 and % EE as Y4 as response parameters [Kesisoglou *et al*., 2007]. The responses obtained from the 29 experimental runs (F1 to F29) were fitted to various models in statistical design [Selvaraj *et al*., 2019]. Among all 29 batches (F1 to F29), F18 was found to have minimum PS (Y1), ZP (Y2) in range, minimum PDI (Y3) and maximum % EE (Y4) (results were shown in section 6.4) which was further selected for validation. The obtained polynomial equations for responses Y1, Y2, Y3 and Y4. These polynomial equations further helped in generating 2D and 3D response surface plots and perturbation plots for PS, ZP, PDI and % EE.

Table 9:Variables and their levels in the Box-Behnken design

Factors	Levels		
Independent Variables	**Low (-1)**	**Medium (0)**	**High (+1)**
A = Solid lipid (mg)	2.5	3.5	4.5
B = Liquid lipid (µL)	250	350	450
C = Surfactant (µL)	100	200	300
D= Cosurfactant (µL)	50	200	350
Dépendent Variables			
Y1 = Particle size (nm)	Minimize		
Y2 = Zeta potential (mV)	Maximize		
Y3 = Polydispersity index (PDI)	Minimize		
Y4 = % Entrapment efficiency (EE)	Maximize		

5.2.7. Optimization and characterization of 5-FU-NLCs

5.2.7.1. Optimization of 5-FU-NLCs

According to the BBD, a total 29 NLCs prototypes (F1to F29) have been developed and characterized for PS (Y1), ZP (Y2), PDI (Y3) and % EE (Y4), as response parameters. The formulation variables were optimized by graphical optimization and overlay plot was obtained as per experimental design. The optimized batch of 5-FU-NLCs (F30) was prepared and characterized for PS, ZP, PDI and % EE.

5.2.7.2. Characterization of 5-FU-NLCs

5.2.7.2.1. Particle size (PS), Zeta Potential (ZP), and Polydispersity Index (PDI)

The PS, ZP and PDI were measured by zeta sizer (Malvern Zeta sizer, Nano ZS90, UK) for all the formulations (F1 to F30). Each formulation (F1 to F30) was filtered by syringe filter (0.22 µm) to remove any possible impurity and 1 mL of it was added to separate cuvettes and analyzed by zeta sizer [Ghosh *et al*., 2020].

5.2.7.2.2. % Entrapment efficiency (% EE)

All developed formulations (F1 to F30) were centrifuged (REMI RM-12C, Remi Electrotechnic Ltd, Varsi, Mumbai, India) at 10,000 rpm for 30 min. The supernatant (10 mL) was collected and diluted suitably using mobile phase (methanol/OPA). The

amount of free drug was determined by HPLC and % EE was calculated by Equation 6 [Sharma *et al*., 2020b; Selvaraj *et al*., 2019].

$$\% EE = \frac{Total\ amount\ of\ drug - Amount\ of\ free\ drug}{Total\ amount\ of\ drug} \times 100 \quad \text{……Equation 6}$$

5.2.7.2.3. % Drug Loading (% DL)

The amount of drug entrapped was calculated as described in section 5.2.7.2.2. and total weight of 5-FU-NLCs (F30) was noted. % DL was calculated using Equation 7.

$$\% Drug\ loading = \frac{Amount\ of\ drug\ entrapped}{Total\ weight\ of\ the\ formulation} X100 \quad \text{……Equation 7}$$

5.2.8. Surface modification of 5-FU-NLCs with chitosan (CS)

From the above parameters used for characterization (as discussed in section 5.2.7), optimized 5-FU-NLCs formulation (F30) was validated and selected on the basis of minimum PS, ZP in range, minimum PDI and maximum % EE (results were shown in section 6.5.2). Hence, this optimized batch (F30) was selected for further surface modification with CS at different concentrations (0.1-0.9 % w/v) to form CS-5-FU-NLCs (F31 to F35) [Tan *et al*., 2017]. Acetic acid (0.1% v/v) was used to dissolve CS in order to increase cross linking potential. After this, F30 batch was added drop wise to beaker containing CS with continuous stirring (500 rpm) on magnetic stirrer for 30 min [Kean *et al*., 2010, Selvaraj *et al*., 2019]. These batches (F31 to F35) were kept undisturbed overnight for cross linking of CS with 5-FU-NLCs which led to complete surface modification of 5-FU-NLCs with CS.

5.2.9. Characterization of CS-5-FU-NLCs formulations

5.2.9.1. Particle size, Zeta potential, and Polydispersity index

The PS, ZP and PDI of F31 to F35 formulations were measured by zeta sizer (Malvern Zeta sizer, Nano ZS90, UK) as procedure mentioned in section 5.2.7.1 [Rabelo *et al*., 2018; Ghosh *et al*., 2020].

5.2.9.2. % Entrapment efficiency

The % EE for surface modified NLCs (F31 to F35) were determined as per procedure described in section 5.2.7.2.2. [Sharma *et al*., 2020b].

5.2.9.3. % Drug loading

The % DL for F33 batch was calculated as as per procedure described in section 5.2.7.2.3.

On the basis of above characterization parameters, CS-5-FU-NLCs formulation (F33, results were shown in section 6.6) was selected for further characterization.

5.2.10. DSC analysis

The DSC thermograms for optimized formulation 5-FU-NLCs (F30) and final batch CS-5-FU-NLCs (F33) were recored by DSC (DSC 6000, Perkin Elmer, USA) as explained in section 5.2.4.2.2. [Mohanta *et al*., 2018; Selvaraj *et al*., 2019].

5.2.11. FTIR analysis

In order to find characteristic functional groups of 5-FU, 5-FU-NLCs (F30), CS-5-FU-NLCs (F33) and CS as well to confirm surface modification by CS over NLCs, FTIR spectroscopy was carried out by FTIR (Perkin Elmer, India Meditech Technologies India Pvt. Ltd. 8400S) as explained in section 5.2.4.3 [Pharmacopoeia, I. 2018].

5.2.12. Scanning Electron Microscopy (SEM)

The surface morphology and 3D shape of optimized formulation of 5-FU-NLCs (F30) and final batch of CS-5-FU-NLCs (F33) was examined by FE-SEM coupled with EDS detector (Au Sputter Coater JEOL JSM-7610F Plus EDS: OXFORD EDS LN2 free). It produces images of a sample surface by scanning the surface coated with gold under a focused beam of electrons. A thin film of formulation was prepared and coated with gold. Further it was subjected to the sample cavity of SEM and analyzed [Mohanta *et al*., 2019].

5.2.13. Transmission Electron Microscopy (TEM)

TEM was performed to analyze the morphological changes of optimized formulation of 5-FU-NLCs (F30) and final batch of CS-5-FU-NLCs (F33) (JEM2100 Plus Electron Microscope, Jeol, Japan). The sample was prepared by staining one drop of each, F30 and F33 formulation with 1% aqueous solution of phosphotungstic acid having negative charge. These were placed into pioloform-coated copper grid (200 μm) using a micropipette, and this thin film was left 1 h for air drying. This was analyzed under the TEM at 50-80 kV at a scale of 500 nm [Varela-Fernández *et al*., 2022].

5.2.14. *In vitro* studies

5.2.14.1. Drug release

The drug release studies of optimized formulation of 5-FU-NLCs (F30) and final batch of CS-5-FU-NLCs (F33) were performed by dialysis bag technique and results were compared with 5-FU solution [Selvaraj *et al*., 2019]. Prior to experimentation, dialysis membrane (molecular weight cut off 12,000-14,000 daltons and pore size 2.4 nm) was washed with distilled water to remove sulphate ions and soaked in buffer (pH 7.4) overnight for activation [Shaima *et al*., 2016]. The NLCs (2 mL) containing 5-FU equivalent to 0.2 mg/mL from each of batch I (5-FU solution), batch II (5-FU-NLCs, F30) and batch III (CS-5-FU-NLCs, F33) was added into dialysis bag and sealed properly. Each bag was placed in separate 250 mL beaker having 100 mL buffer (pH 7.4) as release medium at 37 ± 0.5 °C on magnetic stirrer (100 rpm). The sample (1 mL) was taken at different time intervals i.e. 0, 3, 6, 12, 18, 24, 36, 42 and 48 h and the same volume of fresh buffer was replaced for maintenance of sink conditions. The % cumulative drug release of all the samples were analyzed by HPLC (n=3) [Sharma *et al*., 2020b]. The graph was plotted between % cumulative drug release vs. time. To understand the release kinetics of formulation, the release data was fitted into various kinetic models viz. zero order, Korsmeyer-Peppas, first order, Higuchi and Hixson Crowell for 5-FU-NLCs and CS-5-FU-NLCs [Araújo *et al*., 2012; Dash *et al*., 2010].

5.2.14.1.1. Drug release kinetic modeling

To discus the mechanistic of drug release, the dissolution data was fitted into various kinetic models viz. zero order, first order, while Korsmeyer-peppas, Higuchi and Hixson crowell for 5-FU-NLCs and CS-5-FU-NLCs [Araújo *et al*., 2012; Paarakh *et al*., 2018; Dash *et al*., 2010].

5.2.14.2. Hen's egg test-chorioallantoic membrane (HET-CAM) model

For ocular irritation study, white leghorn chicken eggs were purchased from M/s Sahota Hatchery Centre, Jalandhar, India and incubated for 10 days at 37 ± 0.5 °C temperature and 75 ± 5% relative humidity (R.H.). On the 10th day, the eggs (n=3) were candled to check viability of the embryos. The inner membrane was removed carefully to reveal the CAM and eggs were divided into four groups (n=3). (n=3). All the groups were treated as described here: Group I received 0.5 mL of positive control (0.1N NaOH); group II received 0.5 mL of 5-FU solution (0.2 mg/mL) in water, group III received 0.5 mL of 5-FU-NLCs (0.2 mg/mL), and group IV received 0.5 mL of CS-5-FU-NLCs (0.2 mg/mL). All formulations were applied directly to the CAM using micropipette. After CAM treatment with group-I to IV formulations, the membrane was checked for vascular changes such as haemolysis, lysis, and coagulation. The reactions were observed after 5 minutes and end of 6 h. Then ocular irritation score (IS, score Irritation category 0-0.9 non irritant, 1-4.9 slightly irritant, 5-8.9, moderate irritant, 9-21 severe irritant) was calculated using the following Equation 8 [Selvaraj *et al*., 2019; McKenzie *et al*., 2015].

$$IS = \frac{301 - Haemolysis}{300} \times 5 + \frac{301 - Lysis}{300} \times 7 + \frac{301 - Coagulation}{300} \times 9$$

……..…Equation 8

5.2.15. *Ex vivo p*ermeability study using goat cornea

The procurement of goat eyeballs was done from M/s Sagar Slaughter House, Bhagat Singh Chowk, Jalandhar City (Regd no-22121662000152) and transported to laboratory under cold (4 ºC) saline conditions. The cornea (5-6 mm) was removed and washed with cold saline. This study was conducted in Franz Diffusion Cell (FDC) and

corneal side was continuously kept in an intimate contact with developed formulations. In batch I, 1.0 mL of 5-FU solution (0.2 mg/mL) in water, batch II, 1.0 mL of 5-FU-NLCs (0.2 mg/mL), and batch III, 1.0 mL of CS-5-FU-NLCs (0.2 mg/mL) were taken in donor compartment. The receptor compartment contained simulated tear fluid (STF), pH 7.4. FDC was kept over magnetic stirrer at 100 rpm and temperature of buffer was maintained at 34 ± 0.5 °C. Samples (2 mL) were taken at different time intervals i.e. 30, 60, 90, 120, 180 and 240 min. and the same volume of fresh buffer was replaced for maintenance of sink conditions. The aliquots were analyzed by HPLC (n=3) and permeation of 5-FU was calculated [Sharma *et al*., 2020b; Seyfoddin *et al*., 2016].

5.2.16. *In vivo* studies

5.2.16.1. Antiangiogenesis study by chorioallontoic membrane (CAM) assay

The *in vivo* CAM assay was performed on white leghorn chicken eggs (4 days old) at M/s Sahota Hatchery Centre, Jalandhar. All the eggs (n=3) were cleaned with 70% alcohol prior to incubation. These eggs were incubated at 37 ± 0.5 °C and at 75 ± 5% RH. Afterwards, the eggs were divided into four groups (n=3). On the 5th day, the upper surface of all eggs were pierced with needle and group I was injected with 10 µL of standard positive control (10 µL pyruvic acid), group II was injected with 10 µL 5-FU solution (0.2 mg/mL) in water, group III was injected with 10 µL F30 (0.2 mg/mL), and group IV was injected with 10 µL F33 (0.2 mg/mL). The formulations were injected into the cavity via opening. Re-incubation of all eggs was done after sealing of openings with adhesive tape. On the12th day, the CAM membrane was examined in order to check them for any sign of angiogenesis [Gatne *et al*., 2016].

5.2.16.2. *In vivo* studies in STZ induced diabetic retinopathy rat model

Prior to conduct of experiment, animal approval (protocol number LPU/IAEC/2021/81) was received for ethical conduct of experiment by Institutional Animal Ethics Committee (IAEC) of School of Pharmaceutical Sciences, Lovely Professional University. The Sprague Dawley male rats (90 days old) with average body weight of 230 ± 14 g were purchased and housed at 22 ± 2 ºC temperature, 50% relative humid-

ity (R.H.) and 12 h light/dark cycle in animal house. All the rats were divided equally into 4 groups (n=8). The control group (Group I) of rats received 0.1M PBS (Phosphate Buffer Saline, pH 7.4), the experimental groups (Group II, III and IV) received injection of streptozotocin (STZ, 35 mg/kg) in PBS via intraperitoneal route. After 72 h of the STZ injection, blood glucose levels (BGL) were monitor to ensure development of diabetes in rats. Both control and diabetic rats were fed with normal diet. Body weight and BGL of each rat was checked at the end of every week for a period of 10 weeks. The ten week period was chosen because it was reported for successful development of DR and its associated complications including astrocyte defects, increased neuronal cell death, microglial cell activation, microvascular leakage in rat models of T1DM [Mahaling *et al*., 2018]. At the end of 10th week, in group-I rats, both left eye (LE) and right eye (RE) were administered with PBS as eye drop, In group-II rats, LE was administered with PBS and RE was administered with 5-FU solution (0.2 mg/mL)] in water. Further, in group-III rats, LE was administered with placebo NLCs and RE was administered with 5-FU-NLCs (0.2 mg/mL). In group-IV rats, LE was administered with placebo NLCs and RE was administered with CS-5-FU-NLCs (0.2 mg/mL). Approximately 80 µL of the respective eye drops were administered once a day at fixed time for 20 days. After 20 days, fundus imaging was performed to ensure DR in live rats. After confirmation of DR, rats were sacrificed on day 21 and eye balls were isolated and analyzed for histopathological examination [Seyfoddin *et al*., 2016].

5.2.16.2.1.Fundus imaging

All rats were kept in dark room for 16 hours (dark adaptation) and atropine sulphate eye drops were administered in each rat of group-II, III and IV. The fundus imaging was carried out using fundus camera (UTAS-3000 ERG system, LKC Technologies) [Deng *et al*., 2021; Bearse *et al*., 2006].

5.2.16.2.2.Histopathological examination

The excised eyeballs was immediately placed in 4% paraformaldehyde (PFA). Fixation of the eye balls were done by enucleation and cutting into two hemispheres to remove the vitreous humor and lens. Then, the tissues were embedded in paraffin

wax. Their tissue sections were prepared using a microtome. The sections were de-paraffinized and hydrated in a gradient of ethanol solutions (100, 95, and 70% ethanol) and stained with hematoxylin and eosin (H&E). The images were captured using a microscope (Leica CTR 5000, Leica microsystems, Germany) [Dong *et al*., 2019].

5.2.17. Accelerated stability studies

The CS-5-FU-NLCs formulation (F33) was kept in stability chamber (REMI CMH 10S, Remi Sales & Engineering Ltd, Mumbai, India) for 6 months at 40 ± 0.2 °C and 75 ± 5% RH. The results were evaluated (n=3) in terms of PS, ZP, PDI and % EE at various time intervals such as, 0, 1st, 3rd, and 6th month [Patil *et al*., 2016].

5.2.18. Statistical analysis

All the experimental data (n=3) were expressed as mean ± standard deviation (S.D.). Statistical assessment of the developed data was accomplished by one way ANOVA using GraphPad Prism version 7.0 (GraphPad Software Inc., CA, USA) [Ahmed *et al*., 2020].

6. RESULTS AND DISCUSSION

6.1. Analytical method development and validation

High Performance Liquid Chromatography (HPLC, HPLC LC-20AD, Shimadzu Co. Ltd., Kyoto, Japan) system consists of delivery pump (LC- 20 AD; Shimadzu, Japan), a detector (PDA-photodiode array) (SPDM20A; Shimadzu, Japan), a 20 µL loop (Rheodyne), and software LC Solution [Garg *et al*., 2018] used for analytical method development and validation. A Nucleodur C18 column (Reverse phase, 250 mm × 4.6 mm, particle having 5 micron size) used as stationary phase and combination of Ortho-Phosphoric Acid (OPA) (0.5%) and methanol having ratio 95:5, v/v used as mobile phase in an isocratic mode of elution [Sharma *et al*., 2020b]. The solvent sample mixture (5-FU solution) and mobile phase passes through a HPLC column (stationary phase) and then into a detector, 5-FU solution was injected under the same conditions and HPLC was run for 20 min [Mujeeb *et al*., 2014]. The RT of 5-FU was found to be 7.2 min in 10 min run time of HPLC. The chromatogram peak of 5-FU sample and 5-FU standard were shown in Fig. 8. and Fig. 9. respectively and it was selected further for method validation as the peak was sharp with no tailing or fronting. The blank having water (without drug) was also injected under the same conditions and HPLC was run for 20 min. in which one peak was observed at 4.405 min which indicated that drug having a very sharp peak at 7.2 min. only [Omar *et al*., 2019].

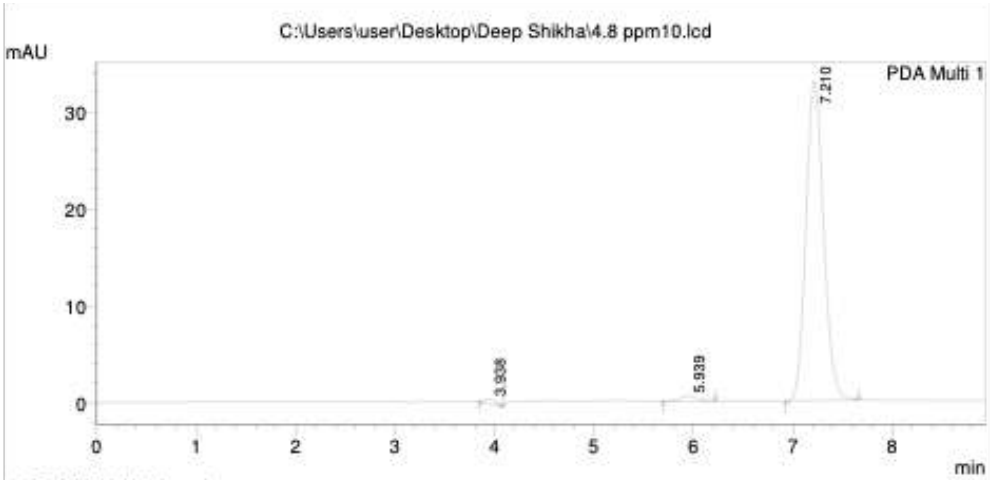


Fig. 8. HPLC chromatogram of 5-FU (sample)

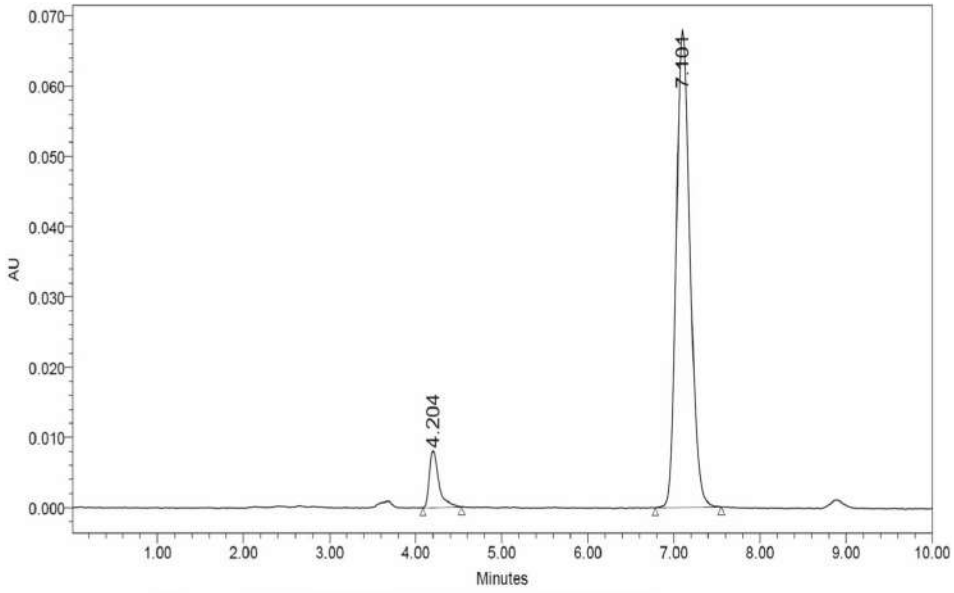


Fig. 9. HPLC chromatogram of (standard)

6.1.1. System suitability

The obtained results of hexaplicate injections 5-FU solution exhibited that the limit tested were the acceptable range. 5-FU was repeatedly retained and well separated at 7.2 min stating very good resolution to specify excellent repeatability of replicate injections on the integral HPLC system used. The number of theoretical plates, tailing factor, peak asymmetry, HETP, and resolution were measured. All the parameters are within the limits presented in Table 10.

Table 10: System suitability parameters

Parameters	**5-FU**
HETP	18.883
Theoretical plate	7943.607
Tailing factor	1.294
Resolution	7.201

6.1.2. Linearity and Range

The graph was plotted between the concentration of drug i.e. 2, 4, 6, 8,10 µg/mL and mean peak area to obtain the calibration curve (n=3) as shown in Fig. 10 [Kamalakkannan *et al*., 2016]. 5-FU calibration curve was linear in the concentration range from 2-10 µg/mL having 0.999 regression coefficient with linear regression equation Y=88843x+13466.

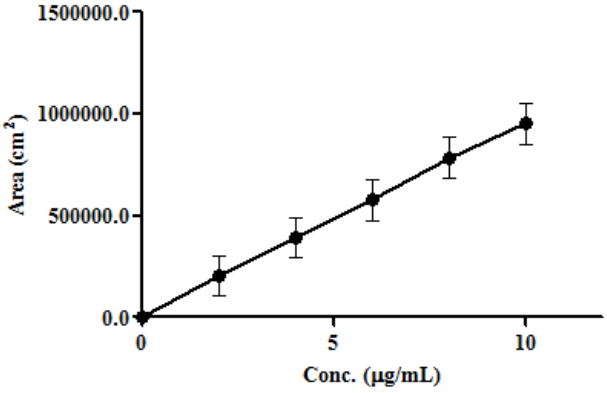


Fig. 10. Standard curve of 5-FU (n=3)

6.1.3. Accuracy

Accuracy was observed by calculating mean % recovery of the drug from HQC, MQC, and LQC solutions containing 4.8, 6.0 and 7.2 µg/mL respectively of sample solution. The data revealed that for all the three levels, the mean % recovery was within the fixed limits of 95-105 % (Table 11). Moreover, the % relative standard deviation (RSD) was < 2%. This indicates the accuracy of the developed method.

Table 11: Accuracy data of the proposed HPLC method from the standard solution of 5-FU

Level	Conc (µg/mL)	Mean peak area (n=6)	% RSD	% recovery	Mean % recovery
LQC	4.8	418793.31	1.16	95.05	96.92
MQC	6.0	520793.81	1.84	95.17	
HQC	7.2	656759.01	1.62	100.56	

6.1.4. Precision

Precision of the method was observed by calculating the % RSD for the 6 injections of the HQC, MQC, and LQC solutions at inter day, intraday and inter analyst by using same experimental conditions. The calculated % RSD for all the samples showing < 2% RSD (Table 12). This proves that the developed method was sufficiently precise. % RSD for the 6 injections of the HQC, MQC, and LQC solutions at inter day, intraday and inter analyst by using same experimental conditions. This proved that the developed method was sufficiently precise.

Table 12: Precision data of the proposed HPLC method from the standard solution of 5-FU (n=6)

Parameters	Levels	Conc. (µg/mL)	1	2	3	4	5	6	Mean area (N=6)	Standard Deviation	% RSD	% Recovery
Repeatability (Intraday precision)												
	LQC	4.8	426409	421031	413124	415398	420817	415981	418793.30	4871.95	1.16	95.05
	MQC	6.0	520272	507850	511819	531648	522721	530453	520793.80	9624.85	1.84	95.17
	HQC	7.2	647909	645153	665459	648195	667899	665939	656759.00	10681.07	1.62	100.56
Intermediate Precision (Interday)												
Day 1	LQC	4.8	427504	427571	421298	420149	420813	420945	423046.70	3498.51	0.82	96.04
	MQC	6.0	578362	577859	567830	575905	576280	578247	575747.20	4013.39	0.69	105.00
	HQC	7.2	641328	623472	632932	635493	632796	621622	631273.80	7456.28	1.18	96.50
Day 2	LQC	4.8	427484	423376	420025	421107	420280	420553	422137.50	2633.26	0.62	95.83
	MQC	6.0	529125	521914	526259	526612	526735	526735	526230.00	2352.26	0.44	96.10
	HQC	7.2	658315	652603	653486	640909	647474	663877	652777.30	8042.18	1.23	99.94

Day 3	LQC	4.8	426409	421031	413124	415398	420817	415981	418793.31	4871.95	1.16	95.05
	MQC	6.0	520272	507850	511819	531648	522721	530453	520793.80	9624.85	1.84	95.17
	HQC	7.2	647909	645153	665459	648195	667899	665939	656759.01	10681.07	1.62	100.56
Intermediate Precision (Inter-analyst)												
Analyst 1	LQC	4.8	422626	425254	418589	422869	411314	416989	419606.81	5065.72	1.20	95.20
	MQC	6.0	576697	578655	567866	577069	576541	553699	571754.50	9636.60	1.68	104.70
	HQC	7.2	668853	657830	657326	639130	651552	644430	653186.81	10593.11	1.62	100.00
Analyst 2	LQC	4.8	421879	422209	424847	422832	420616	421308	422281.80	1467.90	0.34	95.86
	MQC	6.0	526072	529951	528316	514351	523912	518729	523555.21	5967.28	1.13	95.69
	HQC	7.2	625902	622961	627159	622389	624441	624331	624530.51	1782.53	0.28	95.50
Analyst 3	LQC	4.8	417612	424204	423769	420214	429046	415422	421711.21	4956.75	1.17	95.73
	MQC	6.0	535344	524297	522601	531344	527763	515741	526181.71	6906.21	1.31	96.18
	HQC	7.2	658743	663460	664909	652526	662644	661976	660709.71	4502.98	0.68	101.18

6.1.5. Estimation of LOD and LOQ

LOD and LOQ were determined by the slope of standard curve (S) and standard deviation of response (σ). The calculated LOD and LOQ were 0.870277 ng/mL and 2.637202 ng/mL respectively [Kamalakkannan *et al*., 2016].

6.2. Preformulation studies of 5-FU

6.2.1. Physical description of 5-FU

The physical description of 5-FU was examined by organoleptic properties as shown in Table 13:

Table 13: Organoleptic properties of 5-FU

S.No.	Property	Observation	Literature review data	Reference
1	Colour	White	White	https://www.drugbank.ca/drugs/DB00544
2	Odor	Odorless	Odorless	
3	State	Crystalline powder	Crystalline powder	

6.2.2. Melting point determination of 5-FU

6.2.2.1. Capillary fusion method

This method was used to determine melting point of 5-FU using melting point apparatus. The temperature at which the drug starts to melt was noted down (n=3) with the help of thermometer and compared with literature value and tabulated in Table 14.

Table 14: Melting point analysis data

Method used	Literature value	Experimental value (mean ± S.D.)	Conclusion	Reference
Capillary fusion method	282-284 ℃	283.0-284.0 ± 0.5 ℃	5-FU have sharp melting point, crystalline in nature	https://pubchem.ncbi.nlm.nih.gov/compound/3385#section=Chemical-and-Physical-Properties

6.2.2.2 DSC

The DSC thermogram of 5-FU sample and standard is shown in Fig. 11a. and Fig. 11b. respectively. It was evident from the thermogram that the 5-FU shows good thermal stability up to its melting point. The onset melting peak of 5-FU was observed at about 283 ℃ which was close to the reported value as per standard [Tummala *et al*., 2015]. No other characteristic decomposition peak is observed in the DSC thermogram of 5-FU. This indicates that 5-FU was pure, remained stable up to 283 ℃ and do not show any polymorphic form but it undergoes degradation after its melting point.

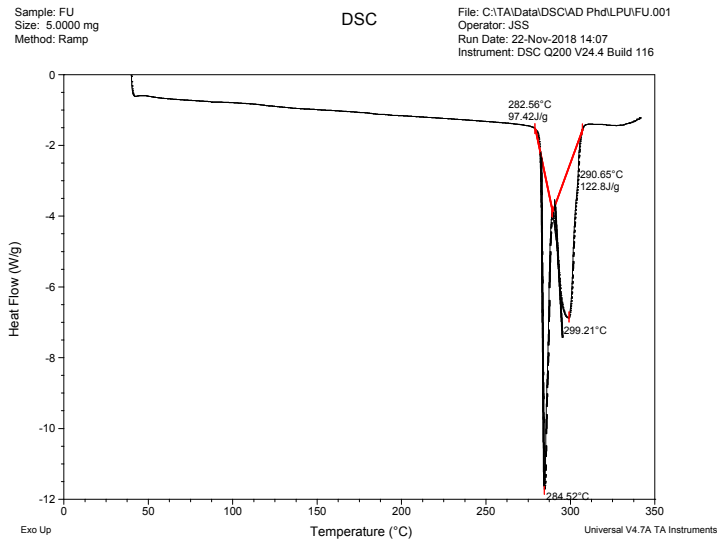


Fig. 11a. DSC thermogram of 5-FU (sample)

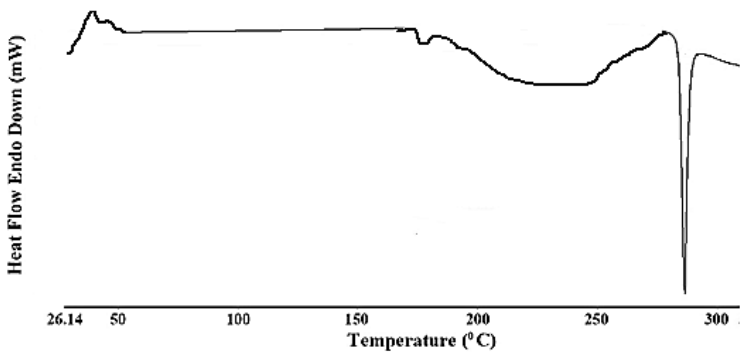


Fig. 11b. DSC thermogram of 5-FU (standard)

6.2.3. FTIR analysis

FTIR scan for 5-FU was taken at wave length 4000 cm^{-1} to 400 cm^{-1} as shown in Fig. 12a. and compared with standard FTIR as shown in Fig. 12b [Pharmacopoeia, I. 2018]. No major significant changes were observed in the absorption peak pattern in comparison with reported value. FTIR spectra of 5-FU showed sharp characteristic peaks at 744 cm^{-1} (CH-CF, aromatic ring), 1498 cm^{-1} (NH^{3+}), 1645 cm^{-1} (NH_2), 2991 cm^{-} (CH_3), and 3064 cm^{-1} (C-C, Alkene) shown in Table 15.

Table 15: FTIR peaks of 5-FU (sample and standard)

S.No	FTIR peaks of sample (cm^{-1})	FTIR peaks of standard (cm^{-1})	Inference
1	3064	3456	C-C (Alkene)
2	2991	2924	CH_3
3	1645	1631	NH_2
4	1498	1551	NH^{3+}
5	744	740	CH-CF (Aromatic ring)

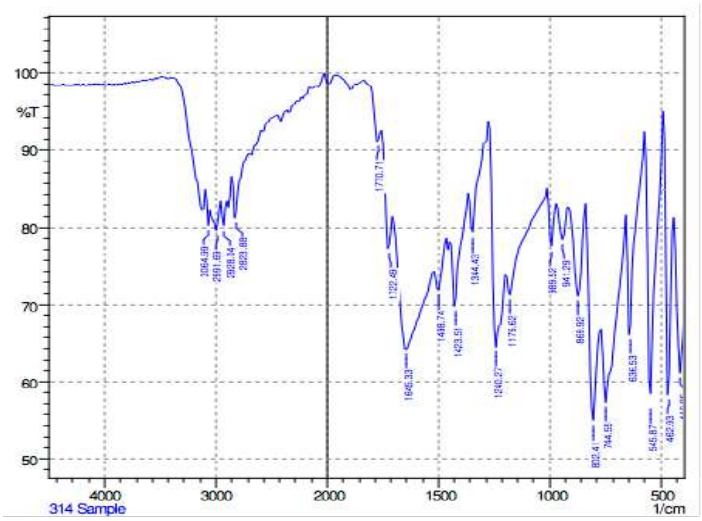


Fig. 12a. FTIR spectra of 5-FU (sample)

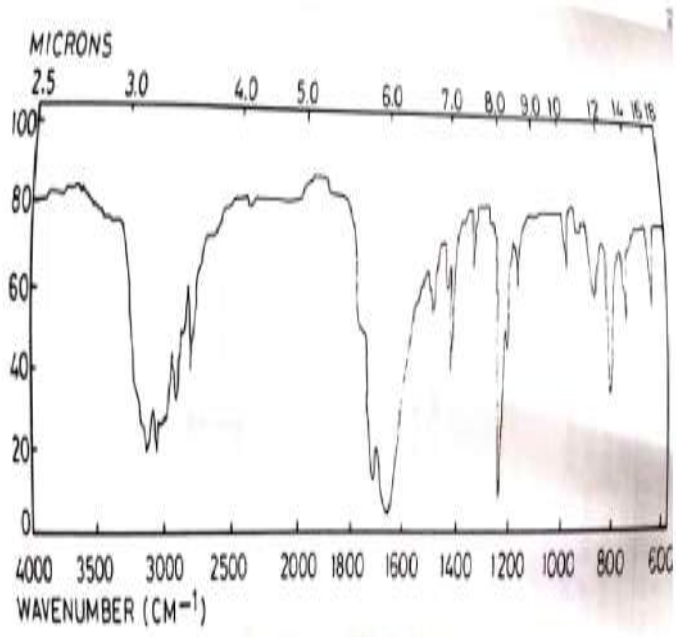


Fig. 12b. FTIR spectra of 5-FU (standard)

6.2.4. XRD analysis

The XRD spectra of 5-FU sample and standard is represented in Fig. 13a. and 13b. respectively. 5-FU shown sharp peaks at diffraction angles (2θ) 16.16, 18.92, 20.51, 21.78, and 28.49 degrees showing a typical crystalline pattern with respect to reported values [Tummala *et al*., 2015]. All major characteristic crystalline peaks, 2θ angle of these peaks and relative intensity remains practically unchanged, which indicated that there was no amorphization of 5-FU and it was still in its original crystalline form.

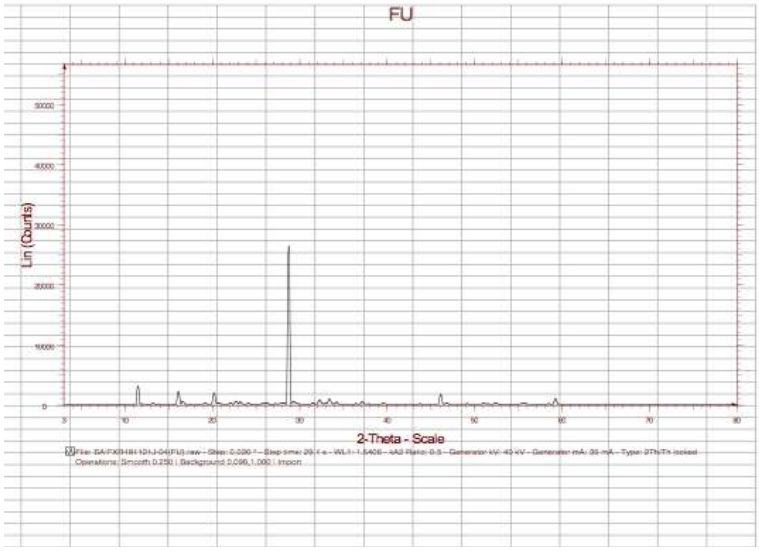


Fig. 13a. XRD of 5-FU (sample)

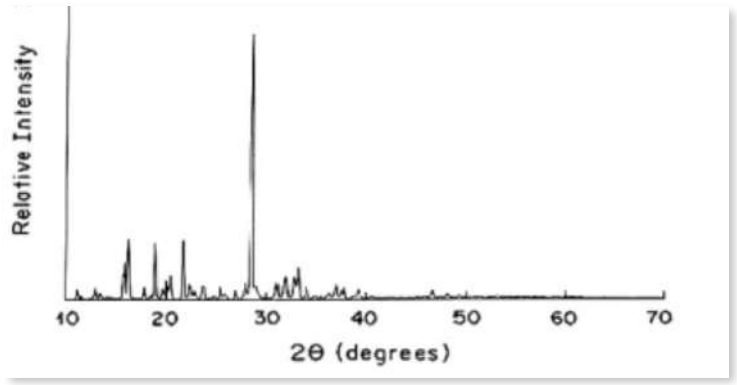


Fig. 13b. XRD of 5-FU (standard)

6.2.5. Partition coefficient determination

The partition coefficient (PC) or log P was calculated (n=3) and results are given in Table 16. The log P was found to be negative which indicated that 5-FU is highly hydrophilic in nature. Moreover, the log P of 5-FU was almost close to the reported value.

Table 16: Partition coefficient (n=3) of 5-FU

Literature value	Experimental value (mean±S.D.)	Reference
-0.87	-0.9 ± 0.23	https://pubchem.ncbi.nlm.nih.gov/compound/3385#section=Chemical-and-Physical-Properties

6.2.6. Solubility studies of 5-FU

The screening of excipients was done on the basis of solubility studies and shown in Fig. 14 below. 5-FU solubility was determined in selected solvents and found to be in decreasing order as follows (Fig. 14a):

Distilled water (97.20 %) > methanol (97.10 %) > PBS buffer (95.20 %) > OPA buffer (93.40 %) > 10% ethanol (93.10 %) > ethanol (88.20 %) > STF buffer (88.00 %) > 10% ethanol (93.40 %).

Among selected surfactants and cosurfactants, the solubility of 5-FU was found decreasing in the following order (Fig. 14b):

Transcutol HP (97.20%) > Tween 80 (95.20%) > Propylene glycol 200 (93.98%) > Tween 20 (93.40%) > PEG 200 (90.38%) > PEG 600 (88.00%) > Propylene glycol 400 (85.50%) > Propylene glycol 600 (40.81%) > Span 80 (12.75%).

The solubility of 5-FU in various liquid lipids and solid lipids was found decreasing in the following order (Fig. 14c):

GMS (96.31%) > LMCS (85.31%) > Labrafac Lipophile WL (80.31%) > Labrasol ALF (75.31%) > Groundnut oil (7.05%) > Soyabean oil (6.52%) > Olive oil (6.36%) > Cotton seed oil (6.31%) > Mustard oil (4.76%) > Almond oil (4.45%) > Eucalyptus oil (2.33%) > Castor oil (1.98%) > Sesame oil (1.40%).

Highest solubility of 5-FU was observed in GMS (solid lipid), LMCS (liquid lipid), Tween 80 (surfactant), and Transcutol HP (cosurfactant), as shown in Fig. 14. GMS produced solvation effect due to presence of its long chain fatty acids, which resulted in its higher penetration into the surfactant chain's layer. This caused improvement in the rigidity of the interface [Leung *et al*., 1987; Makoni *et al*., 2020]. LMCS increased the solubility of 5-FU in NLCs which may resulted in improvement of availability of drug at desired site. In addition, lipid-based formulations are reported to maintain suitable nanometer size range and narrow size distribution, independently of the experimental conditions [Tarsitano *et al*., 2022]. Tween 80 is reported to decrease the interfacial tension between lipids and water to form protective coating around droplets of NLCs which helps in prevention of coalescence of particles. It is pertinent to mention here that the critical micellar concentration (CMC) of tween 80 is 0.012–0.015 mM [Sarheed *et al*., 2020] and its concentration used in the present work was above the CMC to ensure optimum particle size (PS), high drug solubilization, drug loading, and long-term stability. Transcutol HP acts as permeation enhancer as well as increases the solubility of 5-FU [Cirri *et al*., 2018; Makoni *et al*., 2020].

Owing to the above mentioned advantages, GMS (solid lipid), LMCS (liquid lipid), Tween 80 (surfactant), and Transcutol HP (cosurfactant) was further selected for development of 5-FU-NLCs.

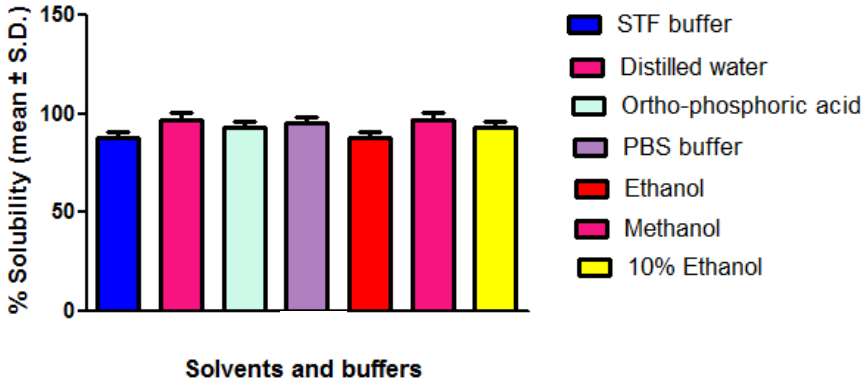


Fig. 14a. Solubility of 5-FU in solvents and buffers

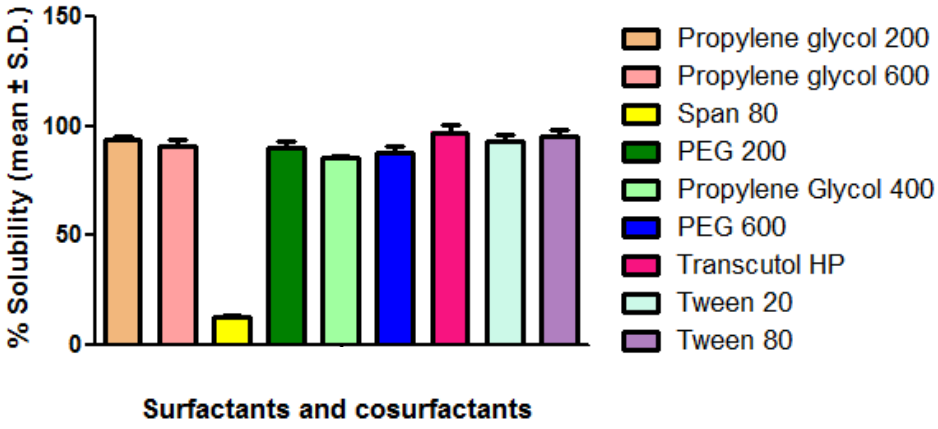


Fig. 14b. Solubility of 5-FU in surfactants and cosurfactants

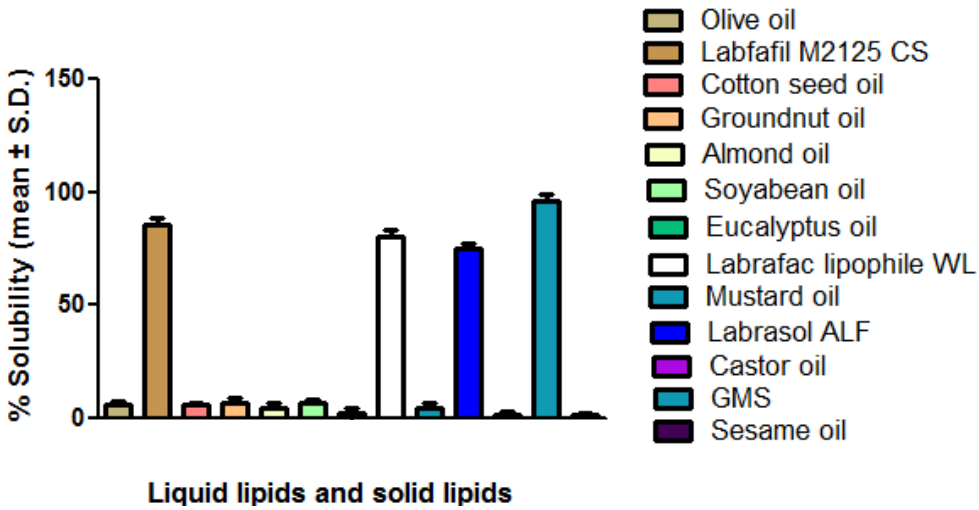


Fig. 14c. Solubility of 5-FU in liquid lipids and solid lipids

6.3. Construction of ternary phase diagram (TPD)

TPD of trial batches (N1 to N81) were constructed and nano-region of TPD was labelled on basis of clear NLCs formed as shown in Fig. 15. The results of TPD showed that few ratios of L_{mix} and S_{mix} shown clear NLCs with no phase separation (N1, N5, N9, N14, N18, N23, N27, N32, N36, N41, N45, N50, N54, N59, N63, N68, N72, N77, N81). It was pertinent to add here that clear NLCs were formed when L_{mix} and S_{mix} were mixed in varying ratios (as per section 5.2.5). Among various NLCs prototypes wherein ratio of L_{mix} was 1:1 and S_{mix} was 1:1, 1:2, 2:1, only six prototypes (N5, N9, N14, N18, N23, N27, as shown in Fig. 15.A) have shown clear NLCs. Same results were obtained for L_{mix} having ratio 1:2, and S_{mix} 1:1, 1:2, 2:1 (N32, N36, N41, N45, N50, N54 as shown in Fig. 15.B). In case where the ratio of L_{mix} was 2:1 and S_{mix} was 1:1, 1:2, and 2:1 total six NLCs formulations (N59, N63, N68, N72, N77, N81 as depicted in Fig. 15.C) were found to be clear. From TPD, it was also observed that different ratios of solid and liquid lipid have significant impact on clarity of NLCs. This indicated that solid and liquid lipid had more impact on PS than surfactants and cosurfactants [Kaur *et al*., 2018].

Out of all the 81 NLCs prototypes, only clear NLCs were selected and characterized for PS, ZP and PDI (Table 17). Their PS was ranged from 110.20 nm (N14) to 251.40 (N63). ZP was ranged from -9.44 mV (N32) to -27.40 mV (N54). PDI was ranged from 0.24 (N14) to 0.45 (N10). The results indicated that higher amount of liquid and solid lipid played a significant role in decreasing the PS of NLCs. Moreover, all these formulations were found stable after 48 h at 40 °C. On the basis of minimum PS, clarity and stablility of NLCs, optimum ZP and minimum PDI, N14 was selected further for preparing 5-FU-NLCs. Results of PS, PDI and ZP for N14 formulation are shown in Fig.16.

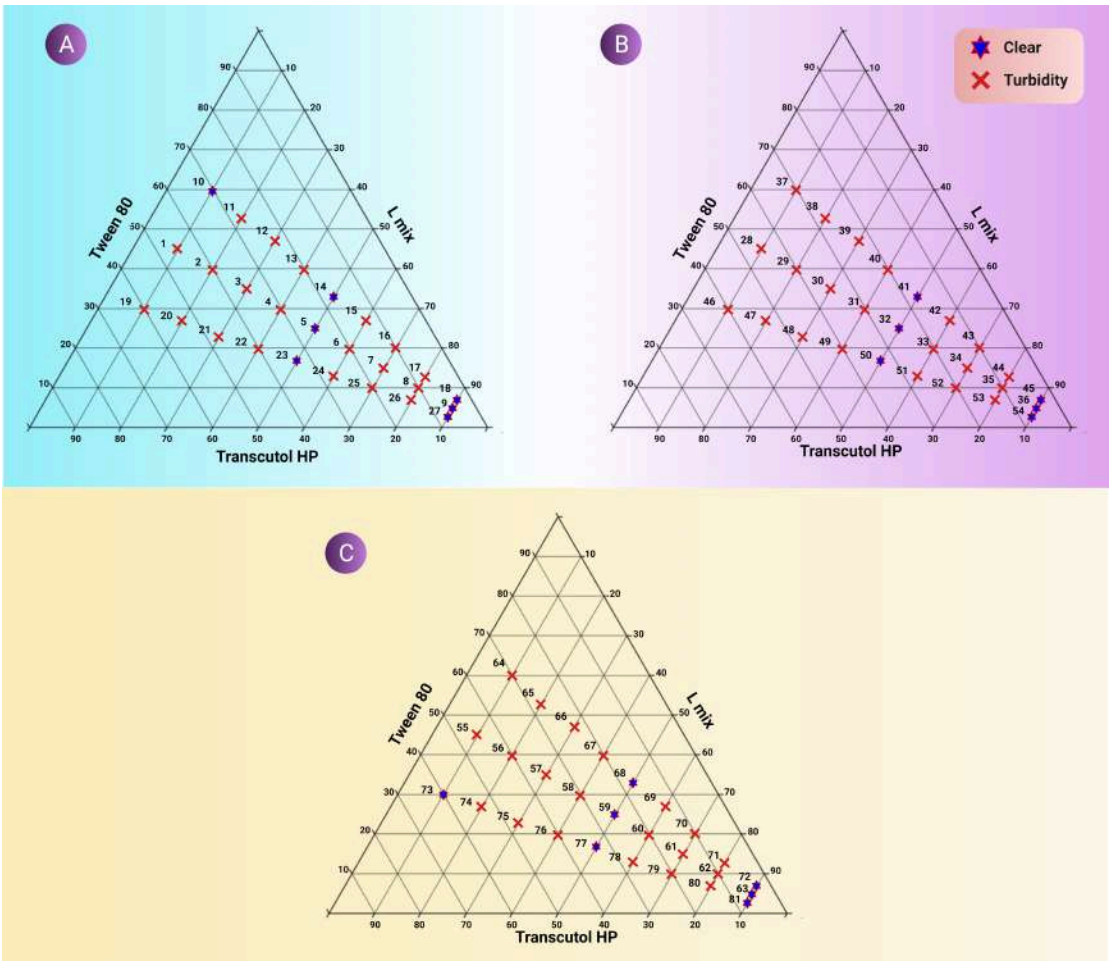


Fig. 15. Ternary phase diagram for 81 NLCs trial batches (A) L_{mix} 1:1 (N1-N27), (B) L_{mix} 1:2 (N28-N54), (C) L_{mix} 2:1 (N55-N81)

Table 17: Particle size, zeta potential and polydispersity index of N1 to N81 formulations as per TPD

Formulation code	Particle size (PS, nm)	Zeta potential (ZP, mV)	Polydispersity index (PDI)
N1	201.40	-9.64	0.428
N5	216.90	-10.10	0.449
N9	163.90	-17.40	0.432
N10	161.40	-16.00	0.453
N14	110.50	-11.30	0.248
N18	120.40	-17.10	0.421
N19	135.70	-19.30	0.261
N23	138.70	-17.40	0.364
N27	136.10	-14.10	0.269
N28	119.50	-13.80	0.379
N32	211.40	-9.44	0.412
N36	222.91	-10.10	0.418
N37	163.31	-15.11	0.422
N41	161.42	-16.01	0.433
N45	151.52	-11.30	0.258
N46	125.92	-17.11	0.429
N50	155.72	-19.03	0.451
N54	148.73	-17.41	0.434
N55	136.13	-14.11	0.359
N59	129.52	-13.81	0.379
N63	251.43	-9.64	0.432
N64	216.92	-10.11	0.428

N68	123.94	-17.11	0.342
N72	161.41	-16.01	0.483
N73	152.52	-11.31	0.248
N77	120.42	-17.11	0.421
N81	135.73	-18.31	0.361

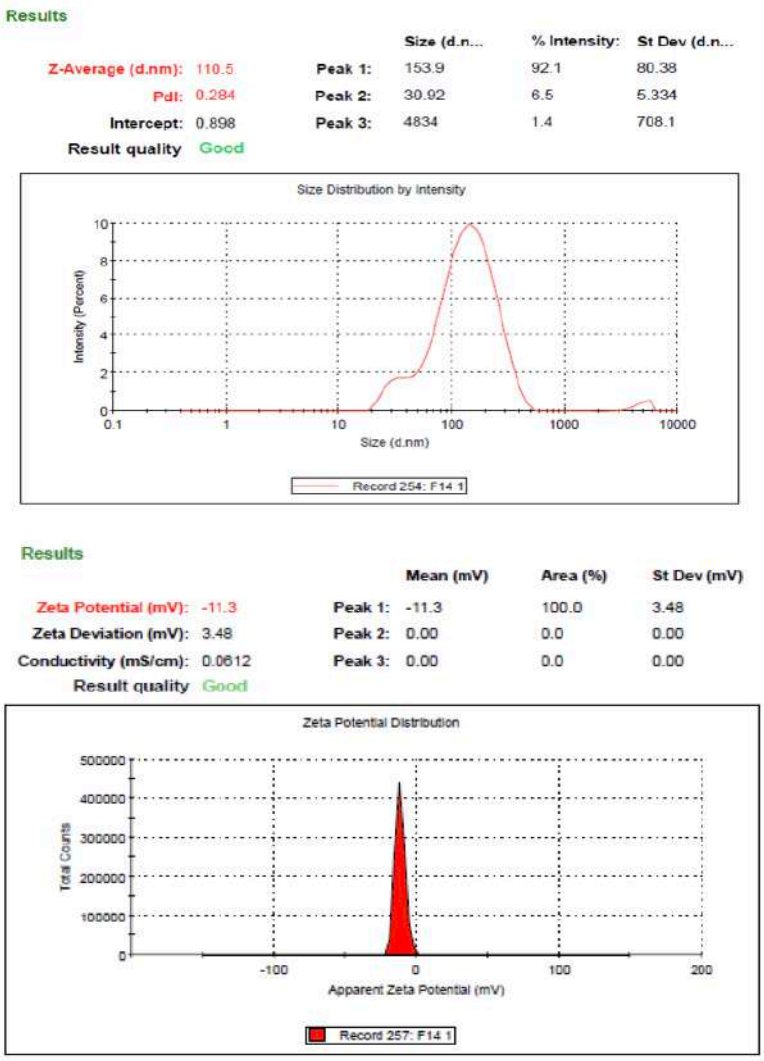


Fig. 16. Particle size, Polydispersity index and Zeta potential of NLCs (N14)

6.4. Screening and optimization of formulation variables

DoE (Design of experiments) tools (sequential model sum of squares, lack of fit test, and model summary statistics) were used for carrying out the statistical analysis of the model used. The lower value of standard deviation (S.D), lower predicted residual error sum of square, high R^2 (coefficient of determination) and $p<0.0001$ suggested selection of quadratic model for responses Y1 (PS), Y2 (ZP), Y3 (PDI), and Y4 (% EE). In all the cases $p<0.05$ indicated that the model was significant and valid. The Analysis of Variance (ANOVA) was used for determining significance and magnitude of independent variables as well as adequacy of model which revealed that responses have responded significantly to the independent variables. The observed responses of 29 batches are shown in Table 18 and top view as well as side view of F1 to F29 batches are shown in Fig. 17a. and Fig. 17b. respectively. The value of R^2 was found more than 0.500 for all the responses. The difference in the values of adjusted and predicted R^2 for responses Y1, Y2, Y3 and Y4 indicated their adequacy to the independent variables. The results obtained for the lack of fit test were found to be non-significant for responses Y1, Y2, Y3 and Y4. The results for ANOVA are summarized in Table 19. Moreover, variance inflation factor (VIF) for responses Y1, Y2, Y3 and Y4 were found less than 1.5, which measures about the inflation in the variances of the parameter and estimates the multicollinearity potential. The obtained polynomial equations for responses Y1, Y2, Y3, and Y4 are given in Equation 9, 10, 11 and 12 respectively. In these equations, positive signs indicated synergistic effect on the responses while the negative signs specified antagonistic effect on responses by factors.

$$Y1=+154.18-1.13A-0.79B+2.75C+2.08D+9.27AB+5.25BC-3.70BD-4.52CD-2.51A^2+4.31B^2+4.31C^2-7.90D^2 \quad \text{Equation 9}$$

$$Y2=-18.79+8.33A-1.23B-0.53C-0.18D-0.75AB-0.60AC-0.17AD-0.35BC+1.44BD+1.35CD-0.054A^2+2.83B^2+0.61C^2-0.26D^2 \quad \text{Equation 10}$$

$Y3=+0.31+5.833A+4.836B+0.041C+0.043D+0.000AB-0.010AC+0.048AD0+0.050BC-9.508BD-0.067CD+0.055A^2+0.051B^2-7.764C^2+0.049D^2$…… Equation 11

$Y4=-74.12+0.33A+0.20B-0.42C+0.28D-2.75AB+2.13AC+0.80AD-2.6BC+4.16BD+2.40CD-2.08A^2+1.22B^2+0.7439C^2+3.52D^2$…… Equation 12

These polynomial equations further helped in generating 2D and 3D response surface plots which showed same effects.

6.4.1. Particle size (PS)

The perturbation plot (Fig. 18A.), 2D contour plots (Fig.19a.), and 3D response surface plots (Fig. 20a.) for PS were plotted. In perturbation plot, it was observed that PS was highly influenced by factor D. The 2D and 3D plots revealed that response Y1 (PS) was directly influenced by factors A (solid lipid concentration) and B (liquid lipid concentration) while C (surfactant concentration) and D factor (cosurfactant concentration) have indirect effect. To check the relationship between excipients and PS, NLCs with different concentration of solid and liquid lipid, surfactant, and cosurfactant were prepared and their PS was measured. During formulation, while decreasing the concentration of solid lipid from 4.5 mg to 3.5 mg and liquid lipid from 450 µL to 350 µL, the viscosity of NLCs also decreased from 10 centipoise to 4 centipoise, which lead to decrease in the surface tension, thus PS got decreased [Bahari *et al*., 2016]. Further, decrease in concentration of tween 80 from 300 µL to 100 µL and transcutol HP from 350 µL to 50 µL caused decrease in the PS. This was due to availability of more amount of surfactant completely covered the oil droplet. The surfactant concentration used in this study was above the CMC to ensure high drug solubilization and loading, optimum PS, and long-term stability [Sarheed *et al*., 2020]. The maximum PS was observed in F21 (161.00 nm) and minimum PS was noted for F18 (129.40 nm).

6.4.2. Zeta potential (ZP)

The perturbation plot (Fig. 18B.) shown that ZP was highly influenced by factor B. As per 2D contour plots (Fig.19b.) and 3D response surface plots (Fig. 20b.), ZP was more affected by A and B factor and less affected by C and D factor. The highest ZP was observed in F24 (-20.50 mV) and lowest ZP in F27 (-13.80 nm). The measured ZP reflected the stability of developed formulations (F1 to F29), in which the physical appearance of NLCs was clear with no signs of instability, such as creaming or cracking. Generally, a ZP greater than ± 30 mV is considered adequate to ensure the physical stability of NLCs [Gurpreet *et al*., 2018]. However, a charge of ± 15-20 mV is sufficient to stabilize the formulation if it is stabilized by both stearic and electrostatic stabilizers [Singh *et al*., 2011].

6.4.3. Polydispersity index (PDI)

In perturbation plot (Fig. 18C.) it was observed that PDI was highly influenced by factor D. The graphs of 2D contour plots (Fig. 19c.) and 3D response surface plots (Fig. 20c.) indicated that PDI has shown positive effect in response to factors A, B, D and negative effect by factor C. The maximum PDI was observed in F2 (0.500) and minimum PDI was observed in F18 (0.210). Salvia-Trujillo and coworkers reported that variation in PDI was due to ultrasound waves used during selected method which provided multimodal distribution at any amplitude or power [Salvia *et al*., 2013]. Also, ultrasounds disrupted larger droplets into smaller ones which led to stabilization of NLCs. This was confirmed by clear appearance of NLCs and absence of phase separation in developed formulations (F1 to F29).

6.4.4. % Entrapment efficiency (% EE)

It was observed that % EE was highly influenced by factor D as per perturbation plot (Fig. 18D). The 2D contour plots (Fig. 19d.) and 3D response surface plots (Fig. 20d.) shown that response Y2 (% EE) was more influenced by dependent variables A and B as compared to C and D. Higher % EE (Y2) was observed due to low surface tension (because of surfactant and cosurfactant) between droplets that prevented their coalescence, which was further confirmed by the absence of phase separation. This led to enhancement in solubility of 5-FU due to presence of GMS containing long chain sol-

id lipid and its retention in NLCs due to presence of liquid lipid, LMCS which avoided leakage of 5-FU [Artiga-Artigas *et al*., 2018]. The maximum % EE was observed in F18 (86.00 %) and minimum % EE was observed in F13 (70.20 %).

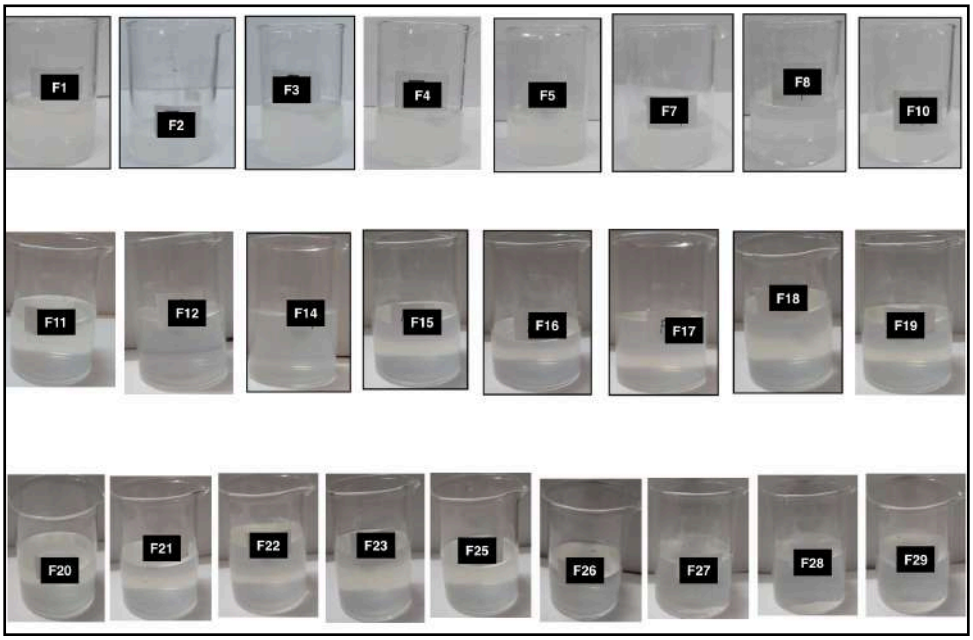


Fig.17a. Side view of DoE formulations F1 to F29

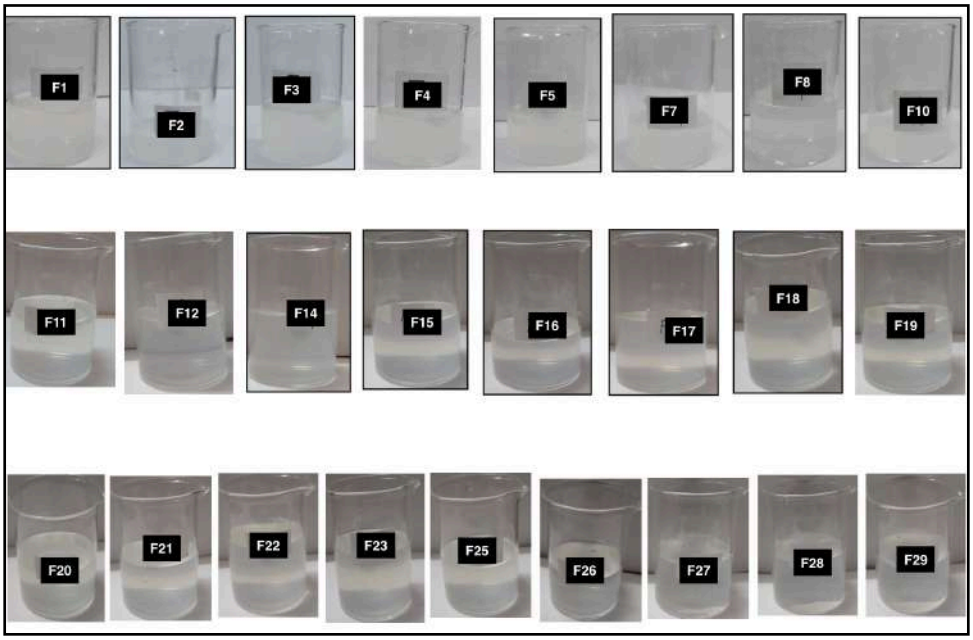


Fig.17b. Top view of DoE formulations F1 to F29

Table 18: Observed responses in BBD matrix for 5-FU-NLCs

Run	Factor 1 A:GMS (mg)	Factor 2 B:LabrafilM2125 CS (µL)	Factor 3 C:Tween 80 (µL)	Factor 4 D:Transcutol HP (µL)	Response 1: Particle size nm (Y1)	Response 2: Zeta potential mV (Y2)	Response 3: PDI (Y3)	Response 4: % EE (Y4)
F1	3.5	450	200	50	146.00	-19.60	0.421	72.80
F2	4.5	350	200	350	146.90	-20.10	0.502	80.50
F3	3.5	350	300	350	143.90	-17.40	0.382	81.00
F4	3.5	350	200	200	155.00	-18.00	0.341	75.40
F5	2.5	450	200	200	143.90	-17.40	0.430	80.00
F6	3.5	350	200	200	155.00	-18.30	0.242	73.20
F7	4.5	450	200	200	160.40	-17.10	0.422	74.90
F8	2.5	350	200	50	143.90	-18.00	0.432	81.00
F9	3.5	350	200	200	155.00	-19.30	0.261	76.00
F10	3.5	250	300	200	148.00	-15.00	0.363	78.00
F11	3.5	350	200	50	146.00	-16.00	0.261	78.00
F12	3.5	450	300	200	155.00	-17.40	0.432	76.00
F13	3.5	350	200	200	155.00	-19.00	0.372	70.20
F14	4.5	350	300	200	145.00	-19.40	0.411	78.30
F15	3.5	350	300	50	147.00	-20.40	0.410	75.40
F16	2.5	350	200	350	141.40	-19.60	0.422	79.40

F17	3.5	450	200	350	144.90	-15.00	0.441	82.30
F18	3.5	350	100	50	129.4	-17.00	0.210	81.00
F19	3.5	350	100	350	144.4	-19.40	0.451	77.00
F20	3.5	450	100	200	142.0	-15.00	0.242	80.00
F21	2.5	250	200	200	161.0	-17.10	0.423	75.40
F22	2.5	350	300	200	156.0	-16.60	0.371	71.60
F23	4.5	350	100	200	143.9	-17.40	0.342	77.00
F24	3.5	350	200	200	148.7	-20.50	0.361	75.50
F25	2.5	350	100	200	146.2	-17.00	0.262	78.80
F26	3.5	250	100	200	156.0	-14.00	0.373	71.60
F27	4.5	250	200	200	140.4	-13.80	0.411	80.60
F28	3.5	250	200	350	155.0	-14.00	0.412	75.40
F29	4.5	350	200	50	142.2	-17.80	0.321	78.90

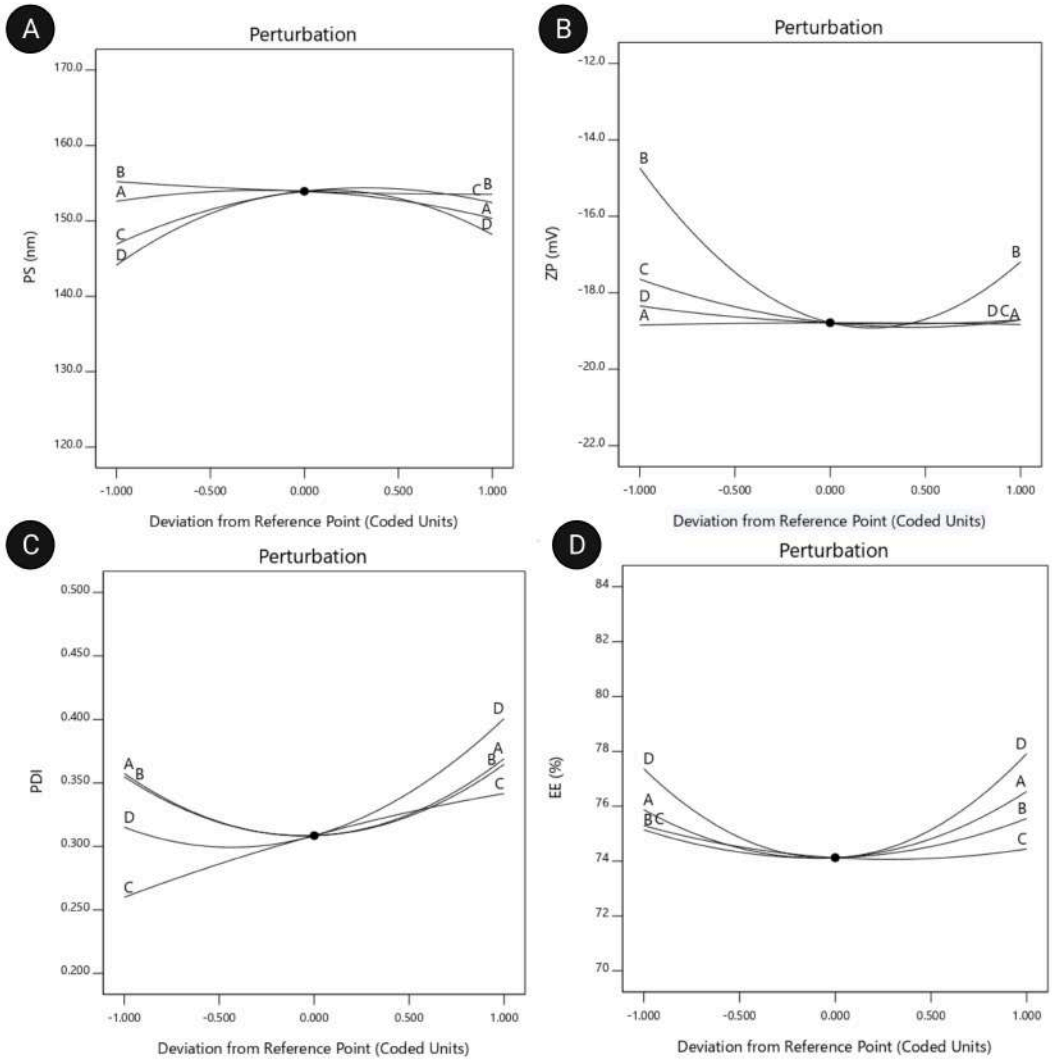


Fig. 18. Perturbation plots for (A) Particle size, (B) Zeta potential, (C) Polydispersity index, (D) % Entrapment efficiency

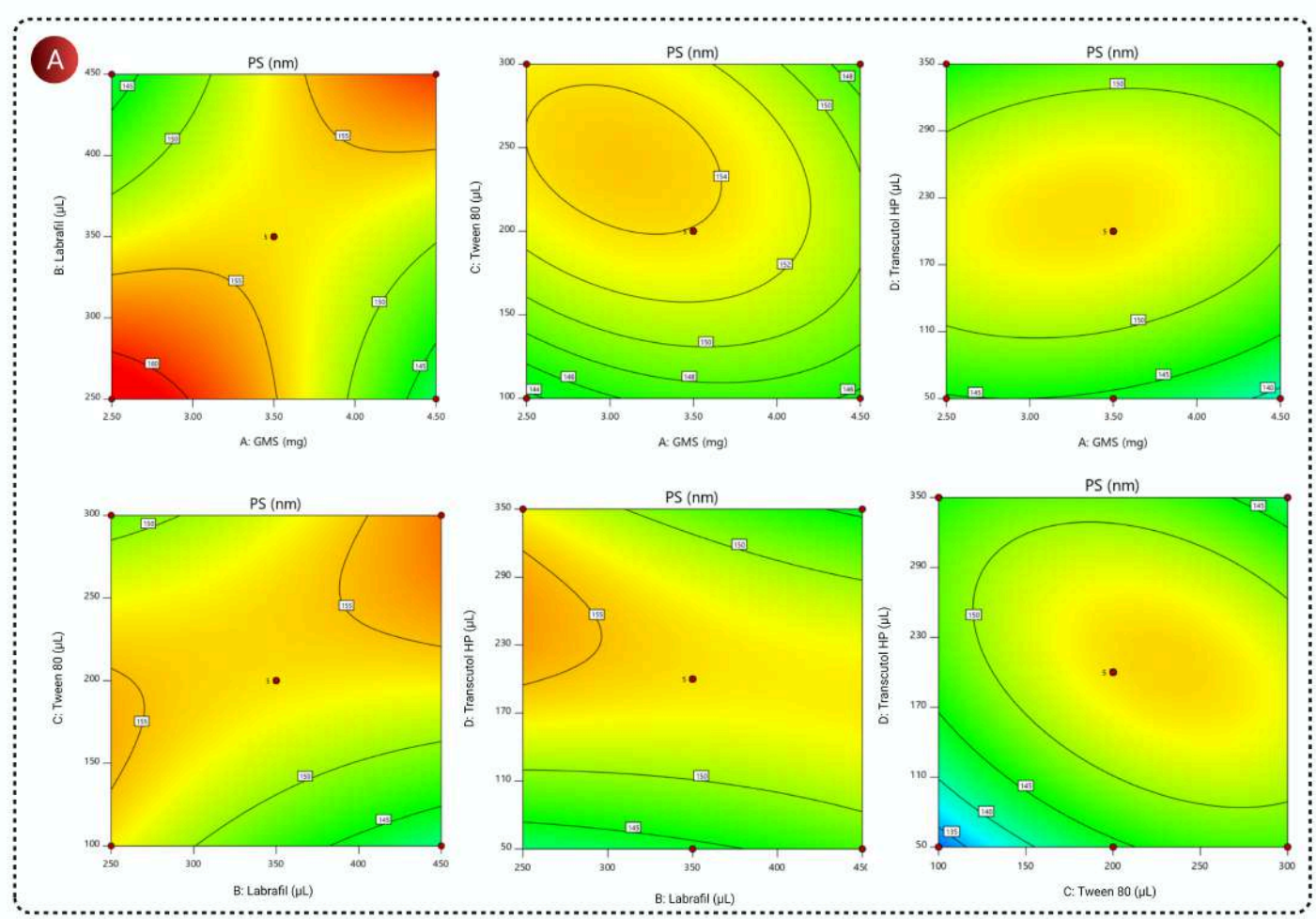


Fig. 19a: Contour plots (2D) of particle size

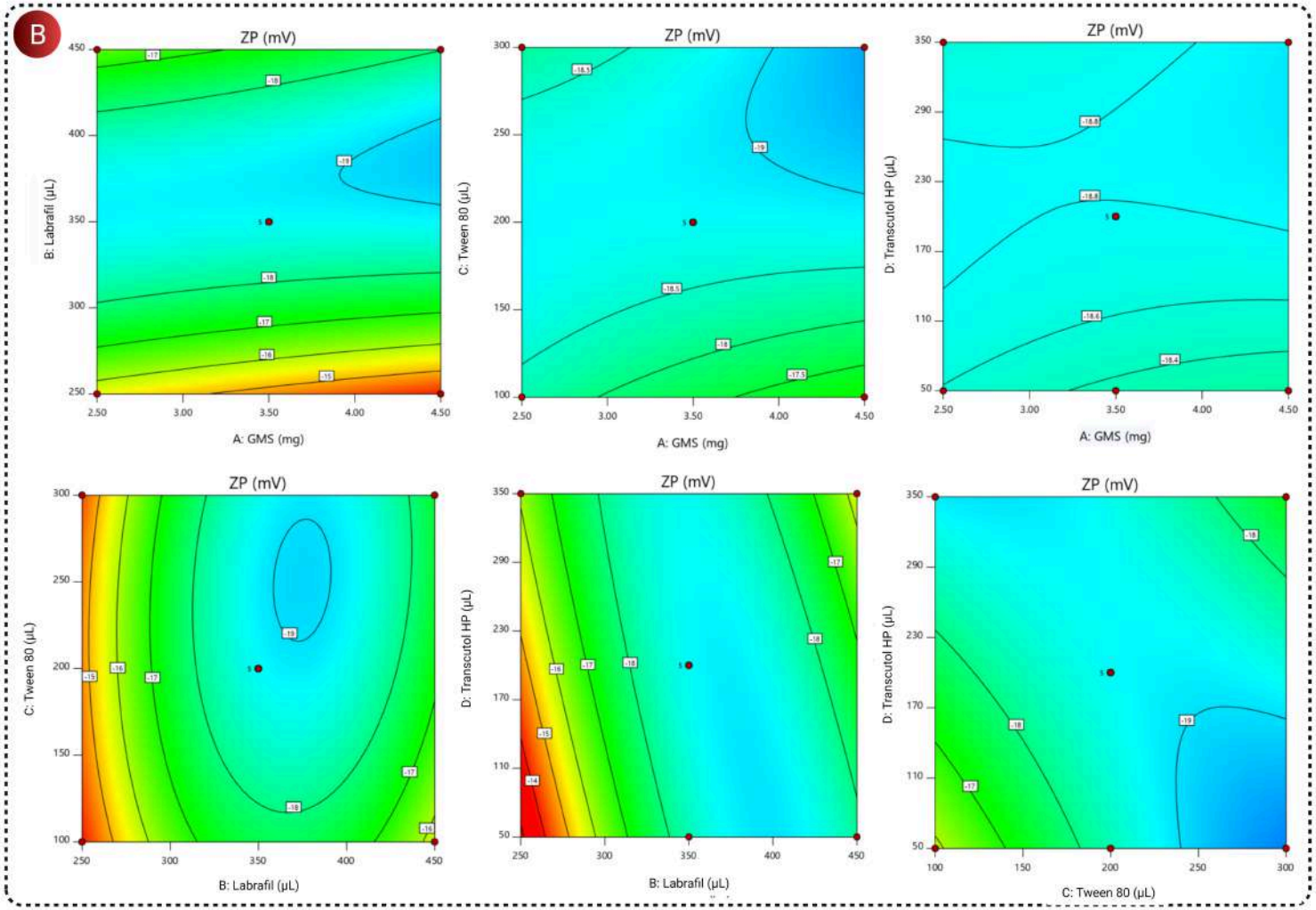


Fig. 19b: Contour plots (2D) of zeta potential

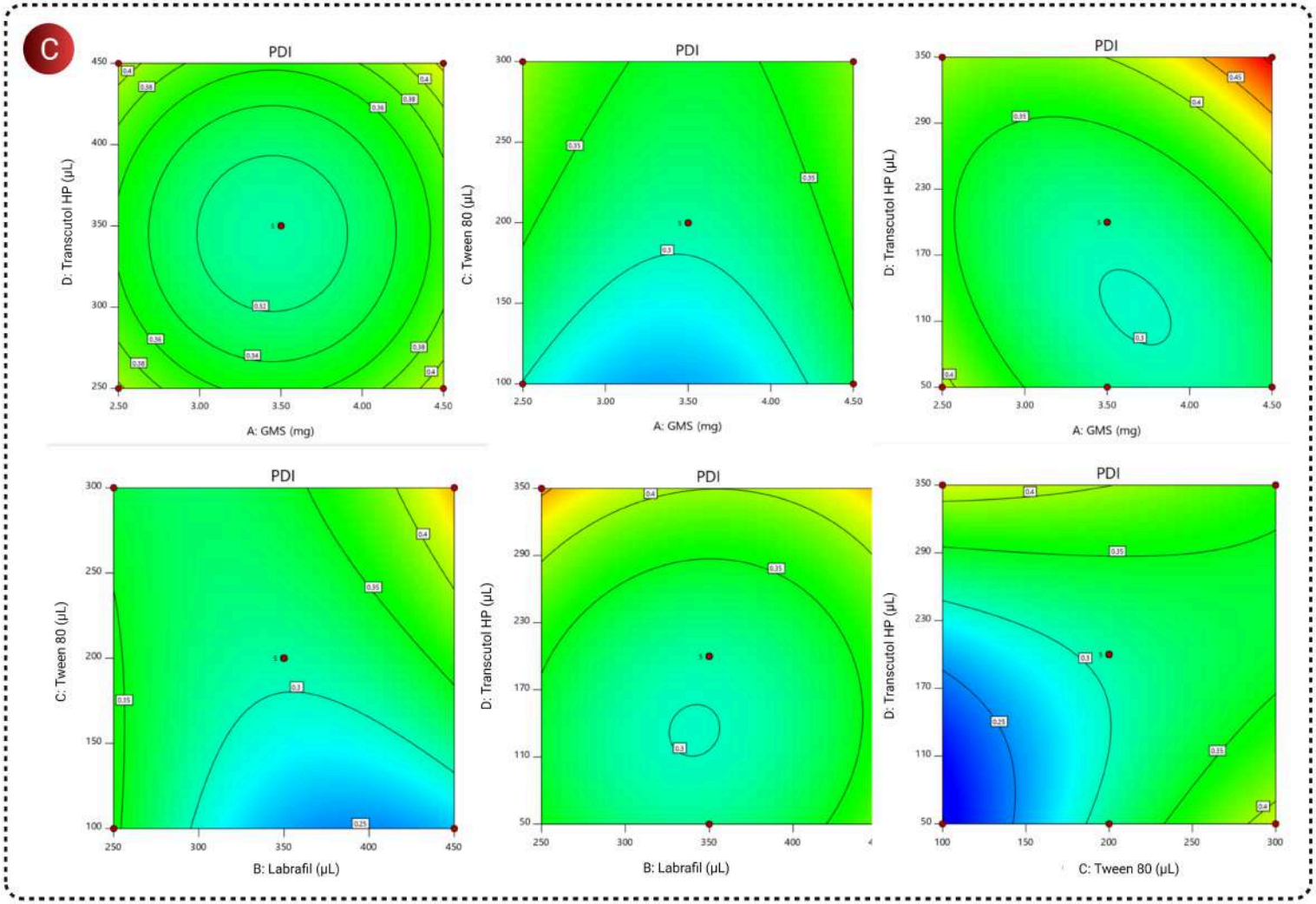


Fig. 19c: Contour plots (2D) of polydispersity index

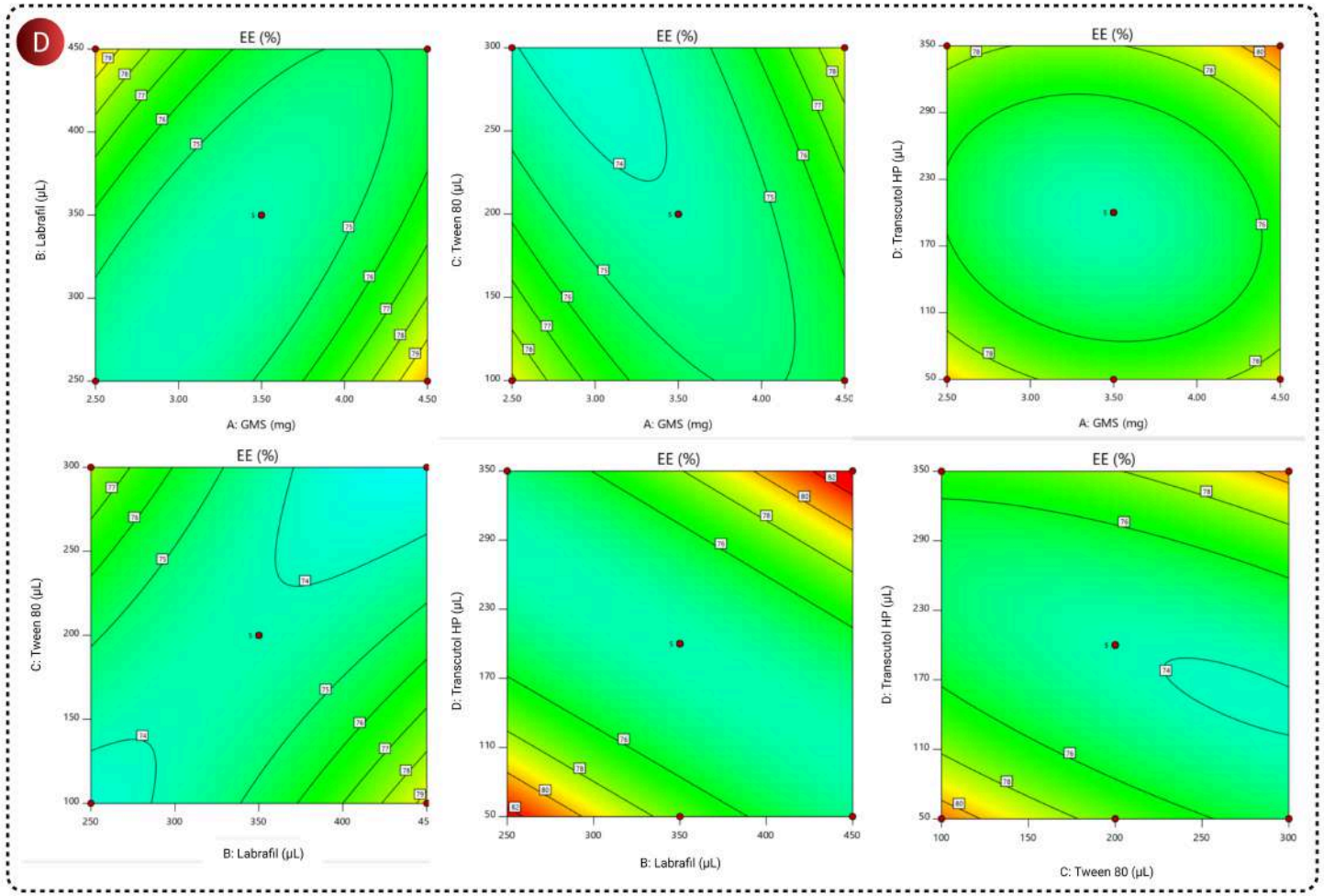


Fig. 19d: Contour plots (2D) of % entrapment efficiency

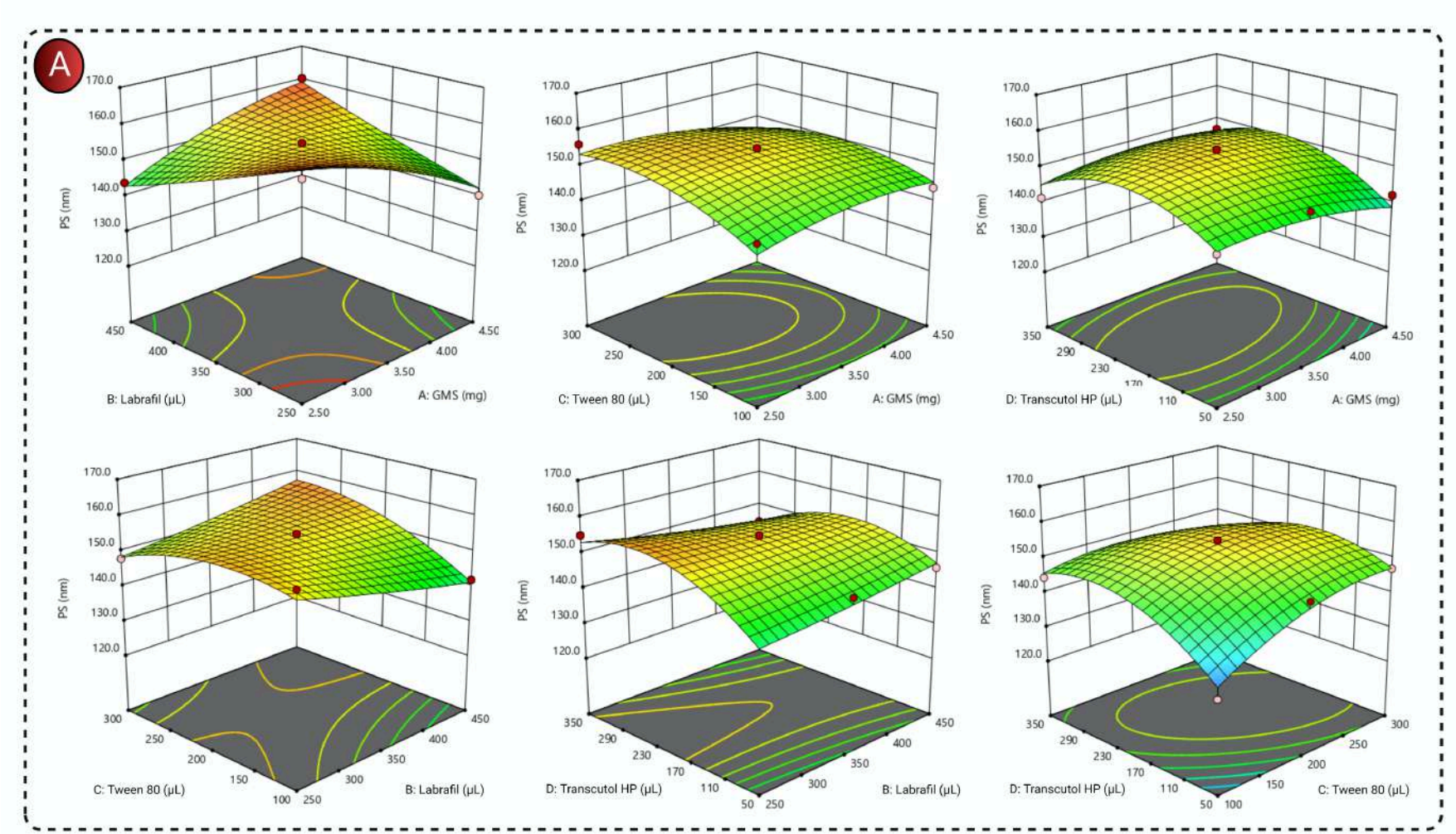


Fig. 20a. 3D response surface plots of particle size

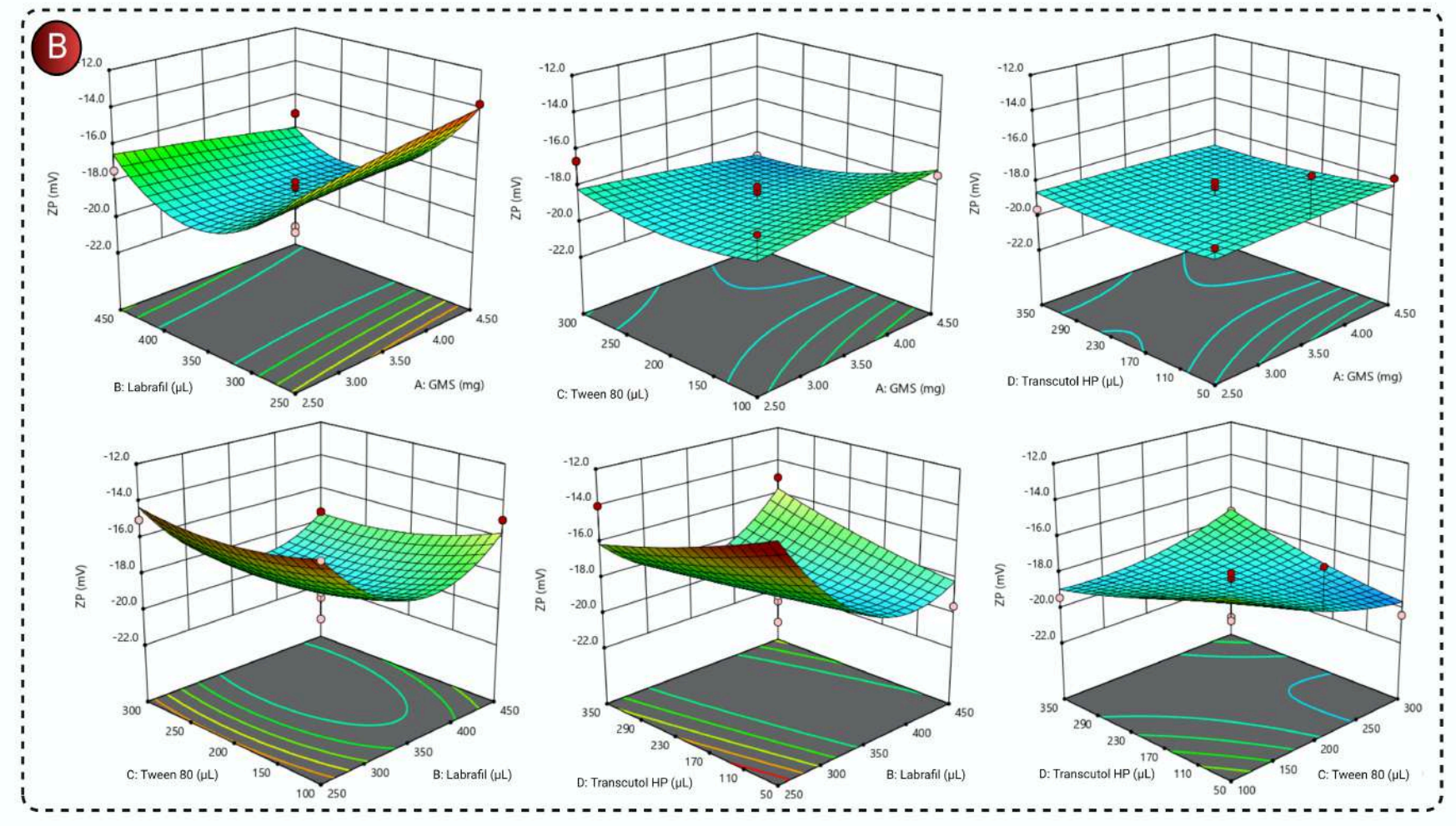


Fig. 20b. 3D response surface plots of zeta potential

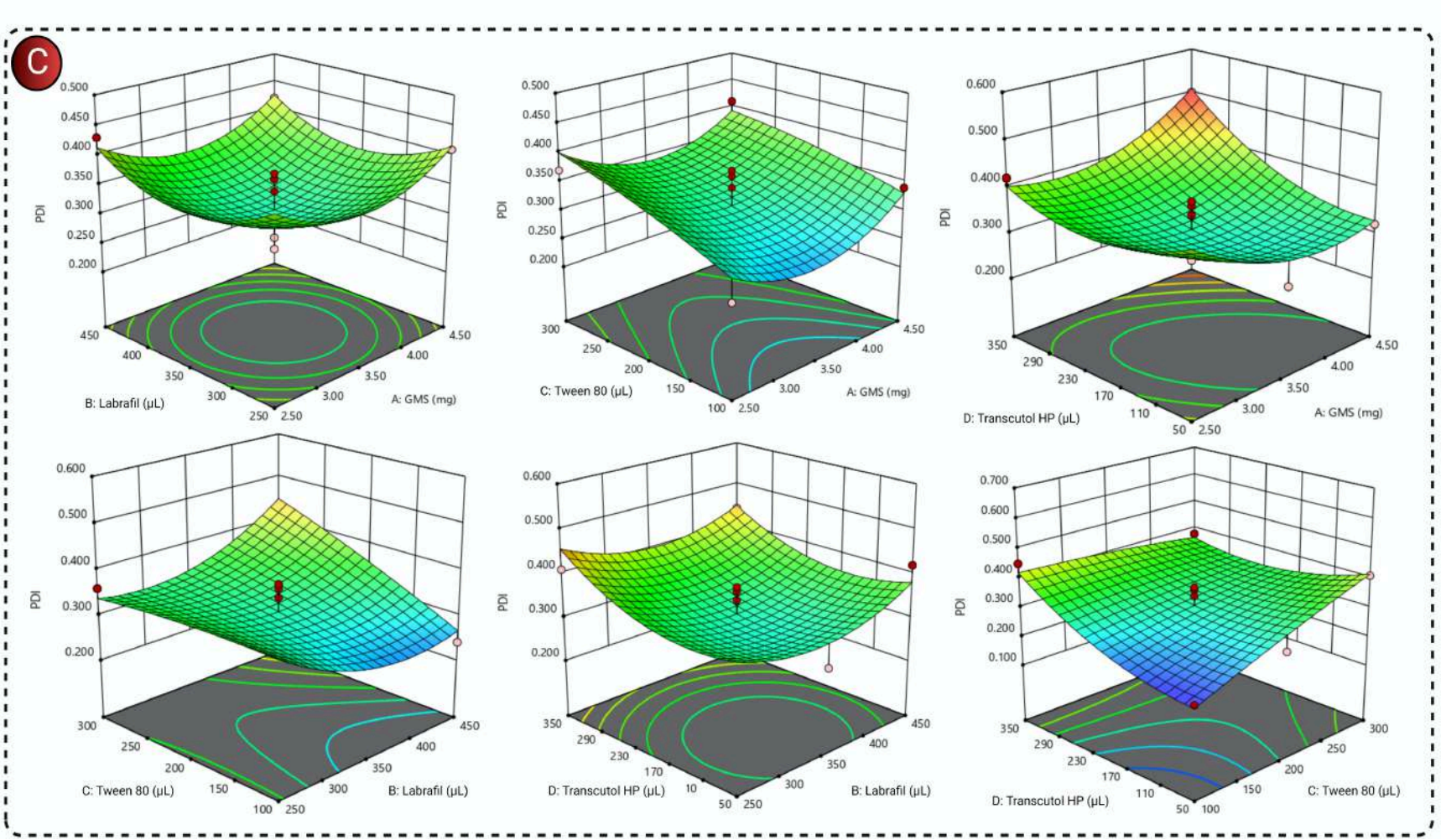


Fig. 20c. 3D response surface plots of polydispersity index

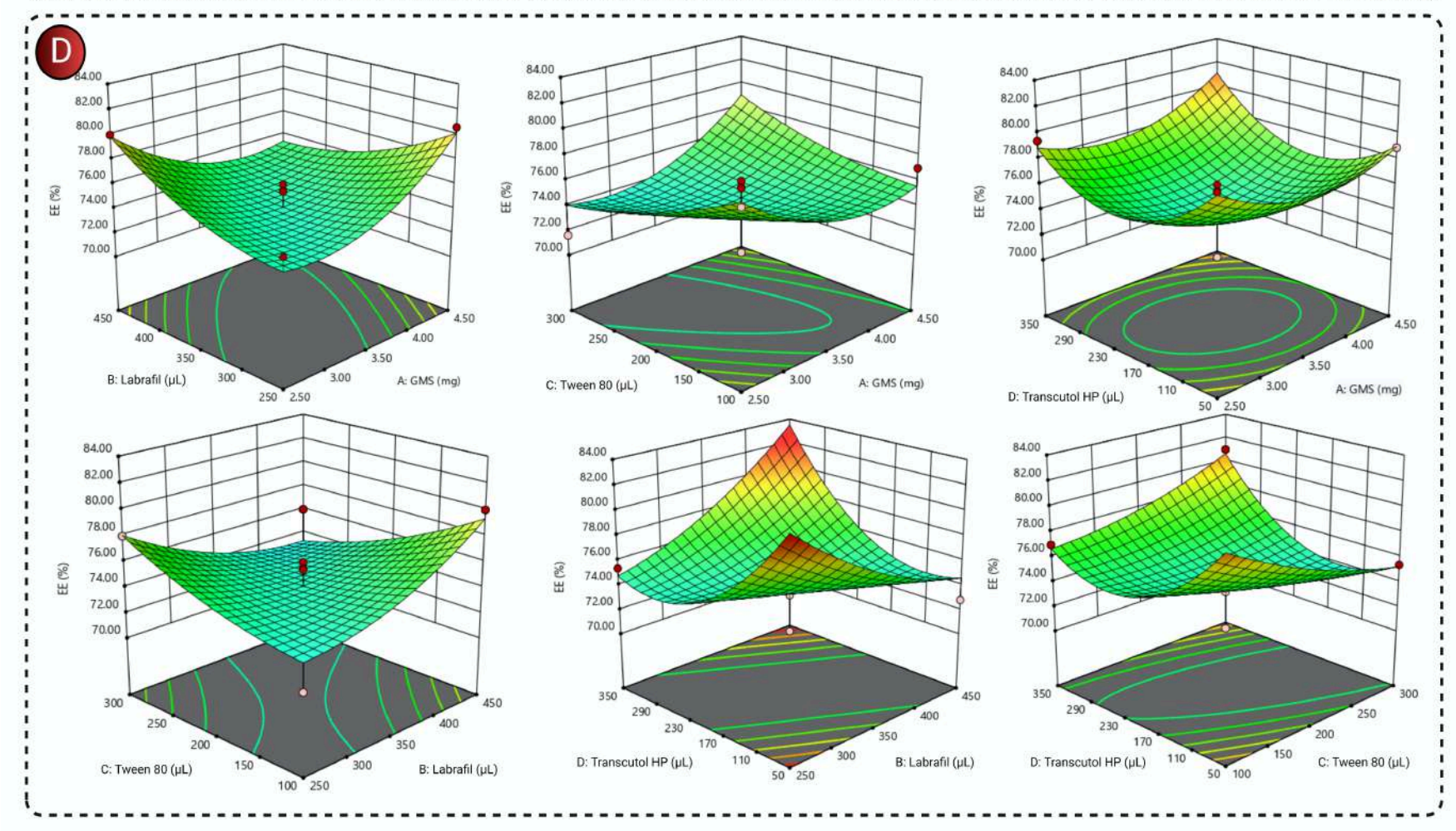


Fig. 20d. 3D response surface plots of % entrapment efficiency

Table 19: Results of fit summary and ANOVA parameters for the measured responses

Sequential Model Sum of Squares [Type I]							
Responses	**Source**	**Sum of squares**	**df**	**Mean square**	**F value**	**p-value Prob > F**	
PS (Y1)	Quadratic	488.47	4	122.12	13.54	0.0001	Significant
ZP (Y2)	Quadratic	50.85	4	12.71	5.78	0.0058	
PDI (Y3)	Quadratic	0.041	4	0.010	5.05	0.0099	
% EE (Y4)	Quadratic	90.48	4	22.62	5.89	0.0054	
Lack of Fit Tests							
Responses	**Source**	**Sum of squares**	**df**	**Mean square**	**F value**	**p-value Prob > F**	Non-significant
PS (Y1)	Quadratic	94.48	10	9.45	1.19	0.4697	
ZP (Y2)	Quadratic	26.98	10	2.70	2.82	0.1649	
PDI (Y3)	Quadratic	0.014	10	1.412	0.39	0.8938	
% EE (Y4)	Quadratic	30.46	10	3.05	0.52	0.8147	
Model Summary Statistics							
Responses	**Source**	**Standard deviation**	**R-Squared**	**Adjusted R-Squared**	**Predicted R-Squared**	**PRESS**	
PS (Y1)	Quadratic	3.00	0.9081	0.8162	0.5543	612.09	
ZP (Y2)	Quadratic	1.48	0.7141	0.4282	0.3826	148.99	
PDI (Y3)	Quadratic	0.045	0.8172	0.6344	0.3941	0.094	
% EE (Y4)	Quadratic	1.96	0.8153	0.6307	0.246	231.43	

6.5. Optimization and characterization of 5-FU-NLCs

6.5.1. Optimization of 5-FU-NLCs

The formulation variables were optimized by graphical optimization. According to the BBD, a total 29 NLCs prototypes (F1to F29) have been developed and characterized for PS (Y1), ZP (Y2), PDI (Y3) and % EE (Y4), as response parameters. The optimized batch (F18) was obtained with a composition of GMS, labrafil M2125 CS, tween 80, and transcutol HP as 3.5 mg, 350 µL, 100 µL, and 50 µL respectively. The overlay plot as per experimental design shown in Fig.21.

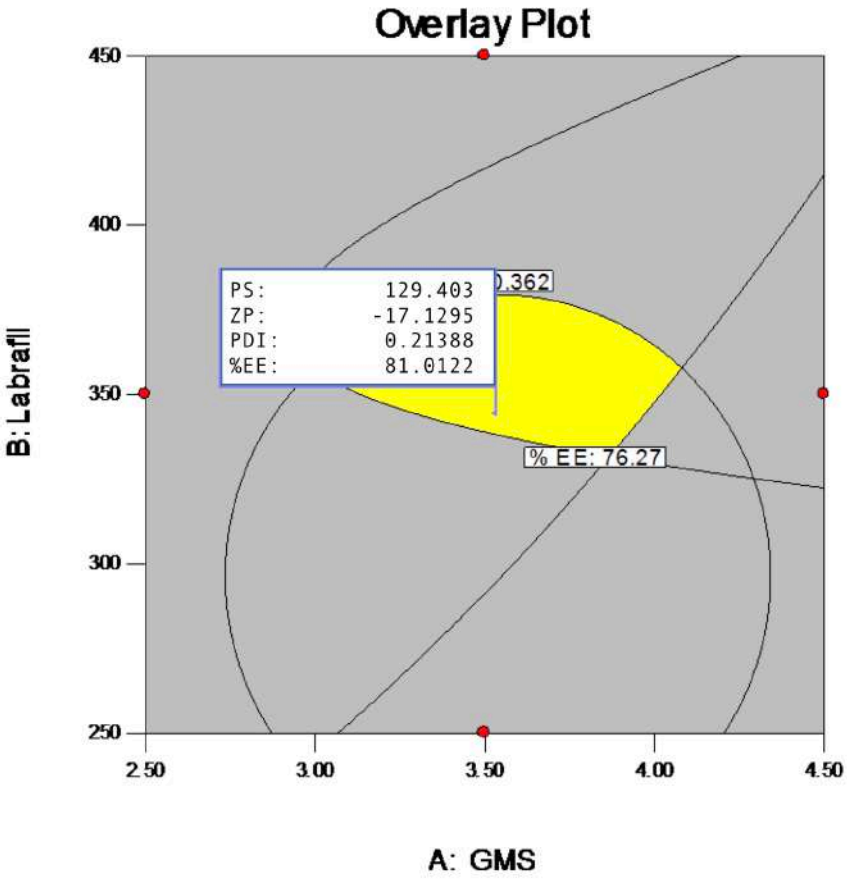


Fig. 21. Overlay plot of optimized batch

6.5.2. Characterization of 5-FU-NLCs

With the use of these predicted values the goal prediction for minimum PS was 129.4 0 nm, ZP of -17.00 mV, minimum PDI was 0.210 and maximum % EE of 81.00 %. The obtained responses of PS, ZP, PDI and % EE for optimized batch (F30) were found to be nano size range (131.10 nm), negative ZP (-16.00 mV), narrow size distribution (0.26 PDI) (Fig. 22) and high % EE (81.40 %) respectively. The % DL for F30 batch was 16.25 %. These values were found in concordance with the predicted values. Hence, formulation F30 (5-FU-NLCs) was used for further surface modification with chitosan (CS) and characterization.

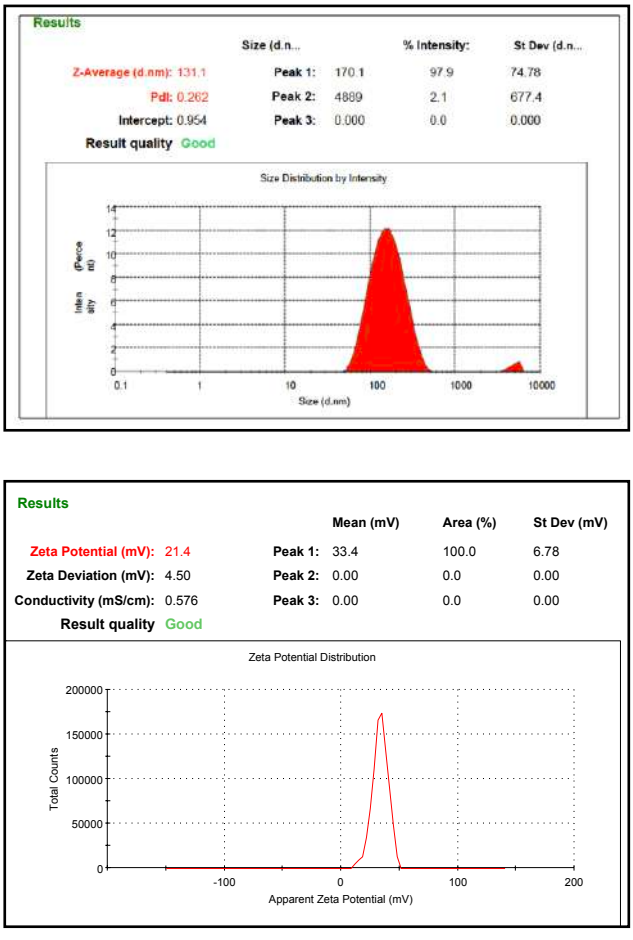


Fig. 22. Particle size, Polydispersity index and Zeta potential of 5-FU-NLCs (F30)

6.6. Characterization of CS-5-FU-NLCs

The surface modification of 5-FU-NLCs (F30) were done with different concentration of CS (0.1 to 0.9 % w/v) in 0.1 % v/v acetic acid to form F31 to F35 batches of CS-5-FU-NLCs (Table 20). All batches were subjected for PS, ZP, PDI, and % EE and observed to be in the range of 163.20 nm (F33) to 168.20 nm (F32) for PS, 19.30 (F31) to 21.40 (F33) for ZP, 0.280 (F33) to 0.430 (F31) for PDI, and 73.30 % (F31) to 85.20 (F33) for % EE. Based on minimum PS and maximum % EE, F33 batch (CS-5-FU-NLCs) was selected in which surface modification of 5-FU-NLCs were done with 0.5% w/v CS. The F33 batch exhibited nano size range (163.20 nm), narrow size distribution (0.280 PDI), positive ZP (21.40 mV) (Fig. 23) and high % EE (85.20 %). The % DL for F33 batch was 17.00 %. Hence, F33 was selected as final batch for further evaluation such as DSC, SEM, TEM, *in vitro, ex vivo,* and *in vivo* studies.

Table 20: Observed responses for CS-5-FU-NLCs

Formulation code	Chitosan concentration (% w/v)	Response 1: Particle size (nm)	Response 2: Zeta potential (mV)	Response 3: PDI	Response 4: % EE
F31	0.1	167.40	19.30	0.431	73.30
F32	0.3	168.20	20.10	0.320	80.20
F33	**0.5**	**163.20**	**21.40**	**0.281**	**85.20**
F34	0.7	165.70	20.70	0.310	75.50
F35	0.9	166.20	21.50	0.430	80.50

Moreover, it was observed that PS was increased in CS-5-FU-NLCs (F33, 163.20 nm) as compared to 5-FU-NLCs (F30, 131.10 nm) owing to surface modification of uncoated 5-FU-NLCs with CS. However, the lower values of PS and PDI (less than 0.5) of both formulations (F30 and F33) are indicative of uniform PS [Hsu *et al*., 2003]. CS cause formation of thick multi-layer on NLCs surface, thus the PS increased after surface modification of 5-FU-NLCs with CS [Bashiri *et al*., 2020] which was further

confirmed by DSC and SEM studies as discussed in section 6.7 and 6.8 respectively and shown in Fig. 24 and Fig. 25 respectively.

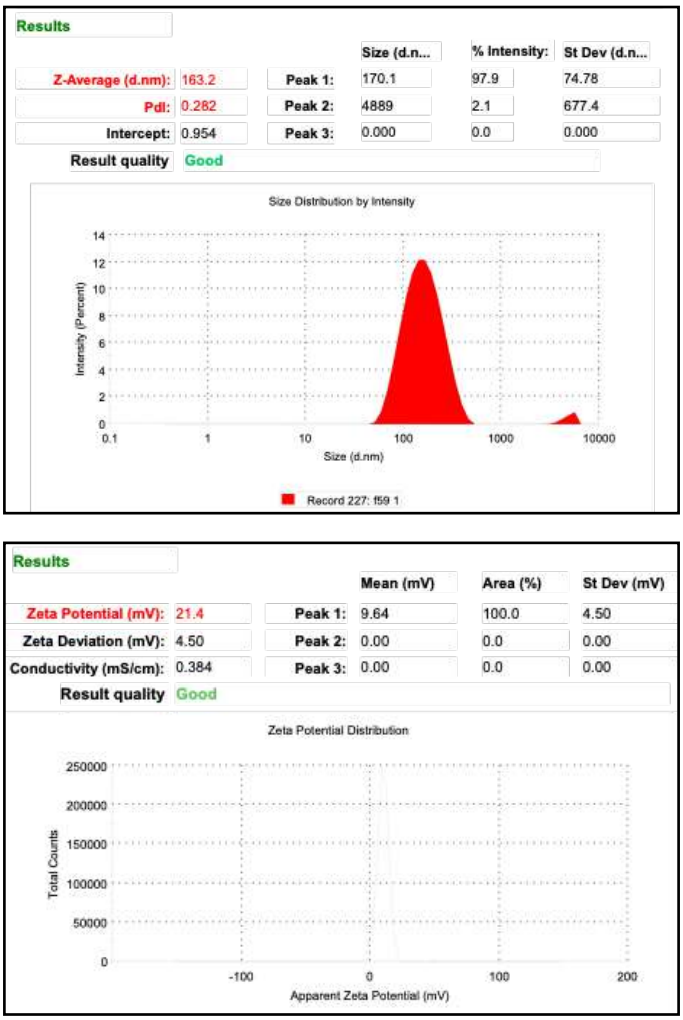


Fig. 23. Particle size, Polydispersity index and Zeta potential of CS-5-FU-NLCs

ZP value also changed from negative (F30) to positive (F31 to F35 batches) due to surface modification of 5-FU-NLCs with CS to form F31 to F35 batches. This also indicated good coating of positive charge of CS on surface of 5-FU-NLCs. CS is a natural polysaccharide with positive charge, having the ability to adhere to negatively charged mucosal eye surface due to electrostatic interactions, which lead to prolonged residence time at drug absorption sites [Nasr *et al*., 2015]. CS has been most widely used for topical delivery of drug to the posterior eye's segment and acted as absorbe-

facient for ophthalmic dosage forms by adhering with negatively charged corneal surface [Kean *et al*., 2010].

Maximum % EE was observed after surface modification of 5-FU-NLCs with CS to form CS-5-FU-NLCs due to increase in PS of 5-FU-NLCs which allowed higher entrapment of 5-FU. Similar findings were reported by Gilani *et al*., for CS coated Luteolin NLCs which shows higher % EE as compared to uncoated luteolin NLCs due to coating with CS [Gilani *et al*., 2021].

6.7. DSC

DSC analysis of 5-FU, CS, optimized batch of 5-FU-NLCs (F30) and CS-5-FU-NLCs (F33) was performed to observe the physicochemical changes occurred during formulation development. In DSC thermogram of 5-FU endothermic peak was observed in 284.52 ºC, but in case of F30 and F33, 5-FU peak was disappeared which indicated 5-FU got solubilized in solid lipid (GMS) and encapsulated inside the lipid matrix. In both batches (F30 and F33) exothermic peak was observed at 69.01 ºC due to evaporation of water from prepared NLCs. In DSC curve of CS, exothermic peak was observed at 300 ºC due to decomposition of amine group and endothermic peak was observed at 100 ºC due to loss of water molecule. DSC thermograms are shown in Fig. 24.

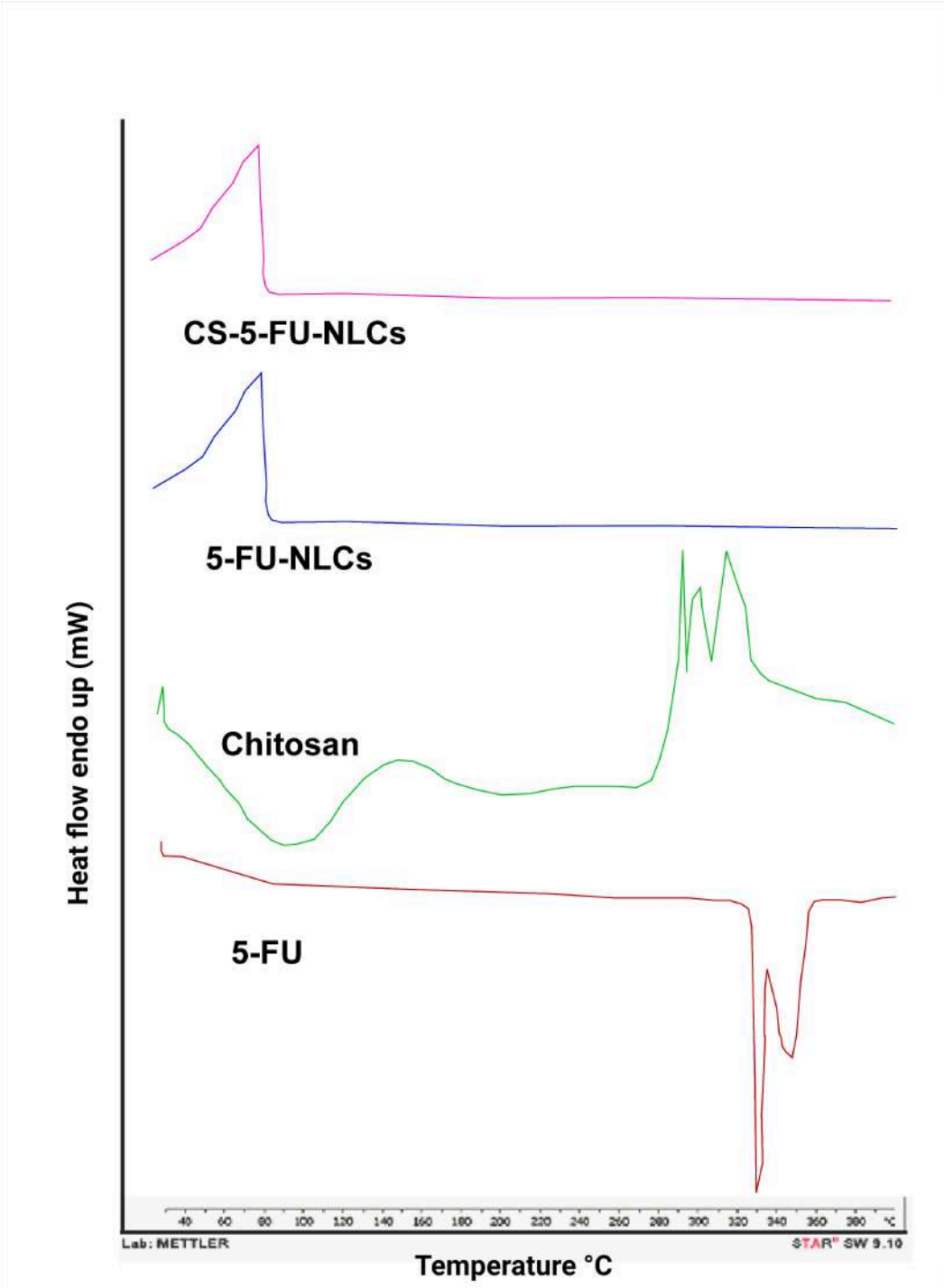


Fig. 24. DSC thermogram of 5-FU, Chitosan, 5-FU-NLCs, and CS-5-FU-NLCs

6.8. FTIR

FTIR spectrum of 5-FU showed sharp characteristic peaks at 3064.99 cm^{-1} (N-H stretch), 1645.33 cm^{-1} (C-N stretch), and 462.93 cm^{-1} (C-H stretch). FTIR spectrum of 5-FU-NLCs also showed characteristic peaks at 3331.18 cm^{-1} (N-H stretch), 1637.62 cm^{-1} (C-N stretch), and 455.22 cm^{-1} (C-H stretch) pertaining to the peaks of 5-FU. There was no significant shift in the vibrational frequencies of 5-FU upon loading into NLCs. This indicated that the drug was compatible with the excipients used to prepare NLCs. However, the obtained peaks were smooth with less intensity, indicating the entrapment of 5-FU in the globules made up of lipid and surfactants [Mahaling *et al*., 2018] . FTIR spectrum of CS showed sharp characteristic peaks at 3429 cm^{-1} (CH_2 stretch), 1652.67 cm^{-1} (C=O stretch), 1380.98 cm^{-1} (C-O-C stretch), and 605.03 cm^{-1} (N-H bend). Similar to the results of 5-FU-NLCs, FTIR spectrum of CS-5-FU-NLCs showed characteristic peaks at 3292.6 cm^{-1} (N-H stretch), 1635.69 cm^{-1} (C-N stretch), 1247.99 cm^{-1} (C-O-C stretch), and 437.86 cm^{-1} (C stretch) pertaining to 5-FU. However, the peaks were smooth with less intensity even as that of FTIR spectrum of 5-FU-NLCs deciphering the coating of CS over the NLCs. Thus leading to surface modification [Deng *et al*., 2021] . The results are shown in Fig. 25 and Table 21.

Table 21: FTIR peak values of 5-FU, Chitosan, 5-FU-NLCs and CS-5-FU-NLCs

FTIR peaks of 5-FU (cm^{-1})	Inference	FTIR peaks of Chitosan (cm^{-1})	Inference	FTIR peaks of 5-FU-NLCs (cm^{-1})	Inference	FTIR peaks of CS-5-FU-NLCs (cm^{-1})	Inference
3064.99	N-H (Stretch)	3429.00	CH_2 (Stretch)	3331.18	N-H (Stretch)	3292.6	N-H (Stretch)
1645.33	C-N (Stretch)	1652.67	C=O (Stretch)	1637.62	C-N (Stretch)	1635.69	C-N (Stretch)
		1380.98	C-O-C (Stretch)			1247.99	C-O-C (Stretch)
462.93	C-H (Stretch)	605.03	N-H (Bending)	455.22	C-H (Stretch)	437.86	C-H (Stretch)

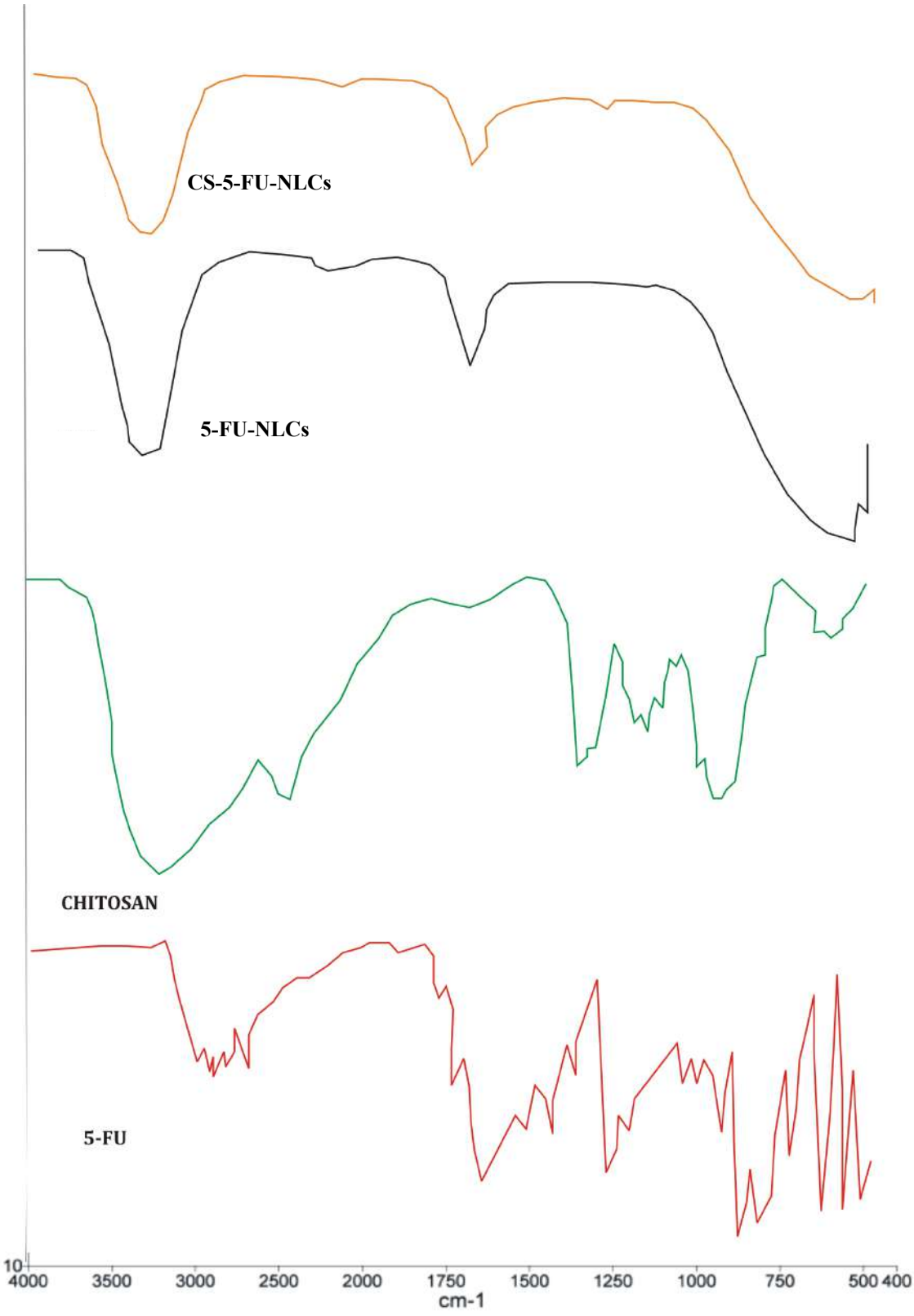


Fig. 25. FTIR spectra of 5-FU, Chitosan, 5-FU-NLCs, CS-5-FU-NLCs

6.9. SEM

The SEM images of optimized batch of 5-FU-NLCs (F30) and CS-5-FU-NLCs (F33) are shown in Fig. 26. The image of 5-FU-NLCs (Fig. 26A.) appeared to be spherical and shown smooth surface with PS scale bar 100 nm. Whereas, in case of CS-5-FU-NLCs (Fig. 26B.) irregular surface was observed due to surface modification of chitosan (CS) on 5-FU-NLCs. The structure of CS-5-FU-NLCs was appeared to be elongated with coarse surface, asymmetrical and smooth boundaries as compared to 5-FU-NLCs. Thus, the outcomes of SEM for developed 5-FU-NLCs and CS-5-FU-NLCs formulation justified the surface modification of 5-FU-NLCs with CS.

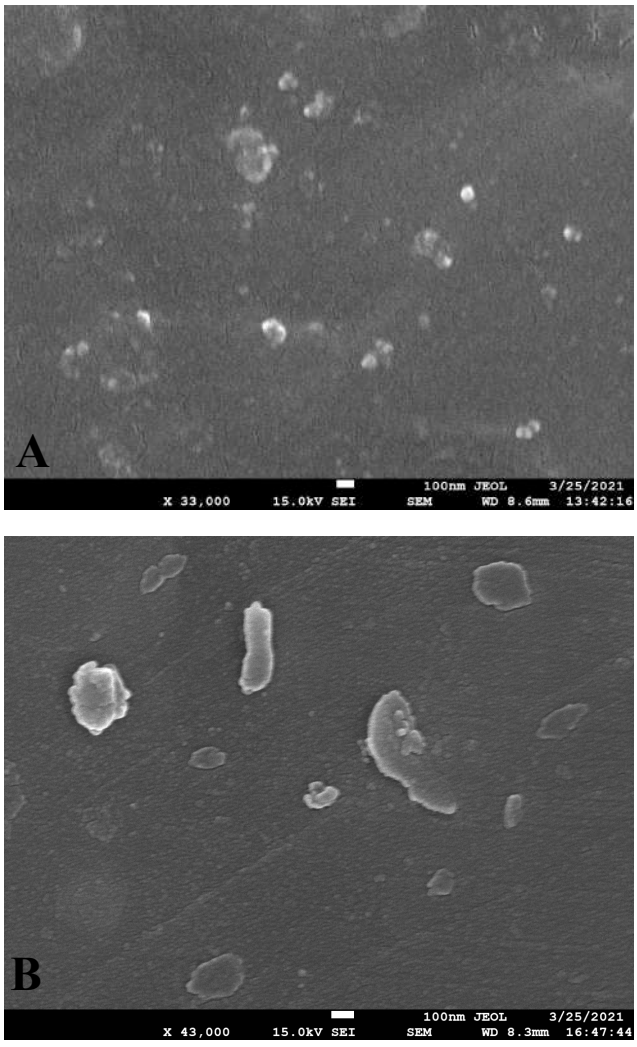


Fig. 26. SEM image scale bar represent 100 nm
(A) 5-FU NLCs (F30) and (B) CS-5-FU-NLC (F33)

6.10. TEM

The TEM analysis of optimized batch of 5-FU-NLCs (F30) and CS-5-FU-NLCs (F33) (Fig. 27) was performed to observe the surface characteristic and morphological observation. In Fig. 27.A. TEM image of 5-FU-NLCs revealed spherical and unagglomerated particles in nanometric range with average diameter of 100.00 nm as per PS scale bar 500 nm. In Fig. 27.B. CS-5-FU-NLCs shown irregular surface particles in nanometer range with average diameter of 180.00 nm size due to surface modification by CS in 5-FU-NLCs formulation.

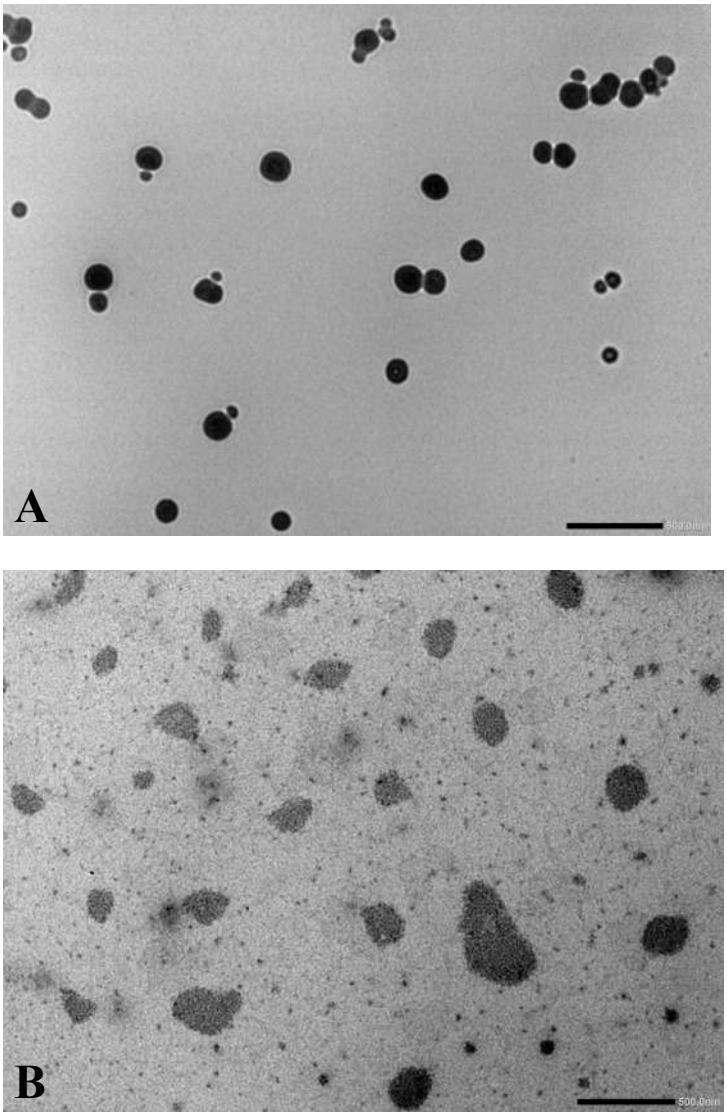


Fig. 27. TEM image scale bar represent 500 nm
(A) 5-FU-NLCs (F30) and (B) CS-5-FU-NLC (F33)

6.11. *In vitro* studies

6.11.1. *In vitro* drug release study

Percentage cumulative drug release (± SD) was calculated (n=3) for batch-I (5-FU solution), batch-II (F30, 5-FU-NLCs), and batch-III (F33, CS-5-FU-NLCs) formulations. The graph was plotted between % cumulative drug release vs. time, as shown in Fig. 28. *In vitro* drug release of batch-I exhibited rapid release of 5-FU with almost 98.50 ± 0.30 % within first 2 h whereas drug release profiles from batch-II and III were found to be sustained and 97.30 ± 0.20 % and 81.00 ± 0.40 %, respectively in 48 h. The results of batch II and III shown sustained release profile of 5-FU over the time period of 48 h as compared to batch-I. However, in case of batch-III comparatively higher delay in drug release profile of 5-FU was observed owing to the swelling property and mucoadhesive nature of chitosan (CS), it form swellable matrix layer around 5-FU-NLCs contributing to controlled drug release viz. diffusion, swelling, and erosion of CS matrix [Khursheed *et al*., 2022; Mohammed *et al*., 2017]. The drug release profiles of batch-I, II and III were compared using one way ANOVA. The p value suggesting significant differences in the release profile of 5-FU in batch-III as compared to batch I ($p<0.01$) and batch-II ($p<0.05$) [Zafar *et al*., 2021]. Similar findings are reported in another study which mentioned that sustained release effect of surface modified NLCs with CS was observed due to encapsulation of drug in the inner core of the lipid matrix which then released slowly by diffusion [Gilani *et al*., 2021].

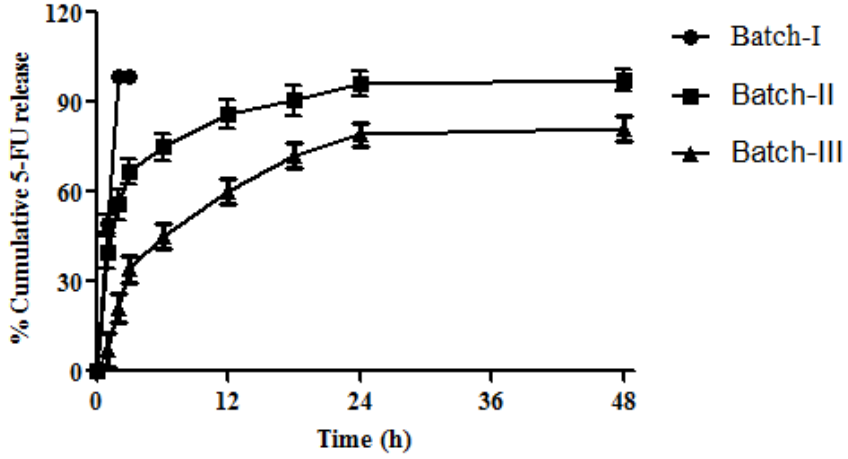
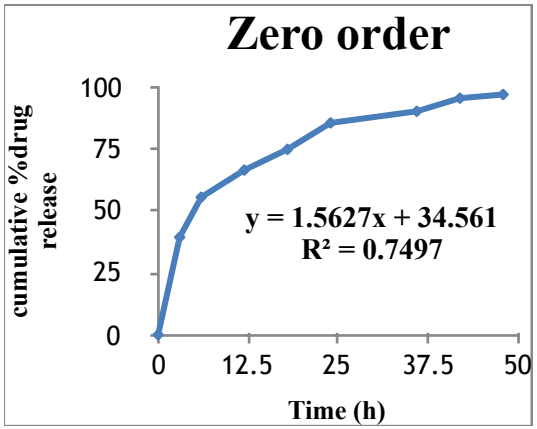
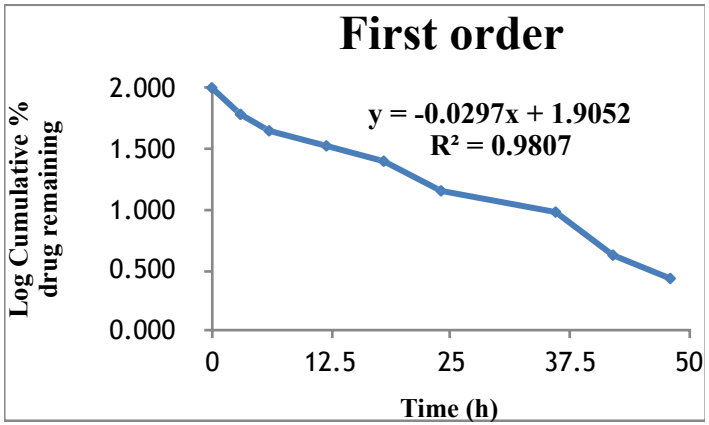


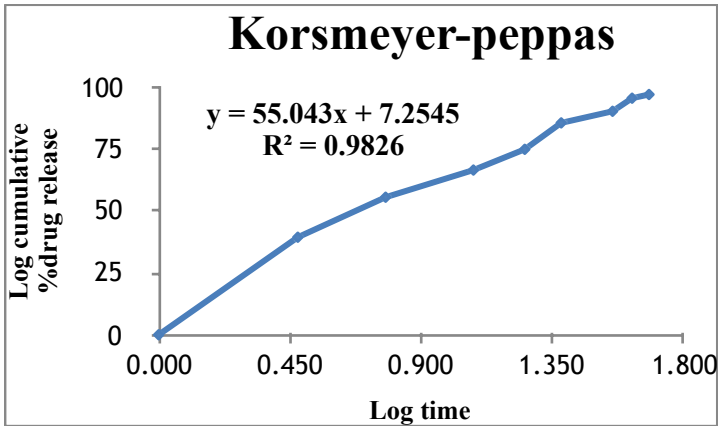
Fig. 28. *In vitro* drug release (mean±S.D.) of batch-I, II and III (n=3) mean ± SD

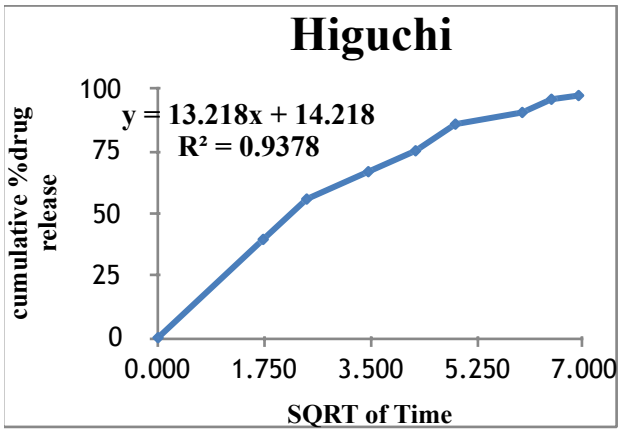
6.11.1.1. Drug release kinetic modeling

The graphs of kinetic modeling for batch-II and III are shown in Fig. 29a. and Fig. 29b. respectively and results are summarised in Table 22, which indicated controlled release of 5-FU from batch-III vis-a-vis batch-II. However, batch-II formulation showed highest R^2 value i.e. 0.978 with Korsmeyer-Peppas model which indicated that 5-FU was diffused from lipid matrix in control release mechanism [Varela Fernandazes *et al*., 2022]. Whereas, in batch-III after surface modification of 5-FU-NLCs with CS, around 1.7 fold augmentation in rate of 5-FU release was observed as compared to batch-II. Also, in batch-III highest R^2 (coefficient of regression) value (0.990) was found with Hixon-crowell model which indicated that release of 5-FU was dependent on change in diameter and surface area of CS-5-FU-NLCs, owing to mucoadhesive property of CS which formed swellable matrix layer around CS-5-FU-NLCs [Lee *et al*., 2014]. The value of kinetic release coefficient (n) was found 0.561 for batch-II and 0.631 for batch-III, thereby exhibiting non-Fickian diffusion owing to drug diffusion and lipid matrix erosion. Hence, it can be concluded that in batch-III, 5-FU release was primarily through diffusion mechanism, plausibly owing to hydrophilic nature of 5-FU. This was followed by complete biodegradation of CS after releasing its contents [Wang *et al*., 2013].









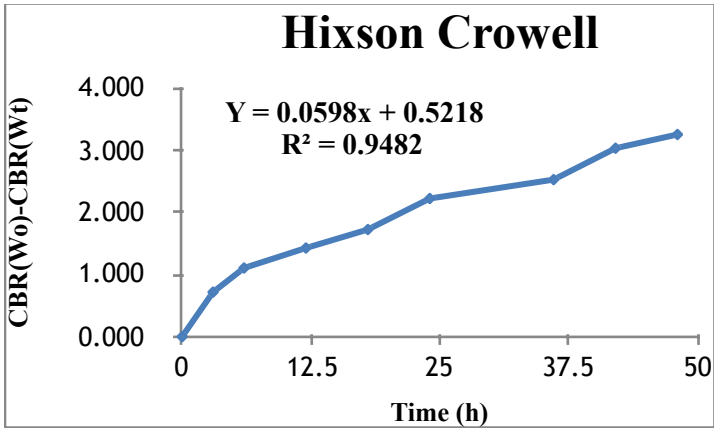
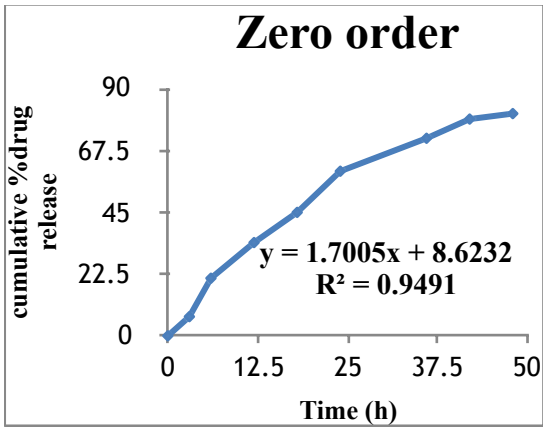
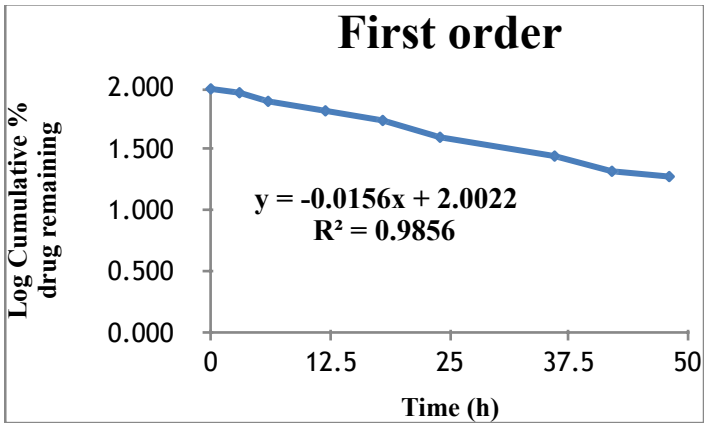
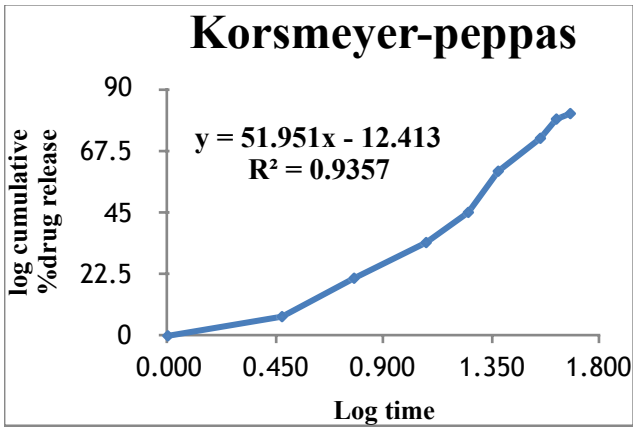
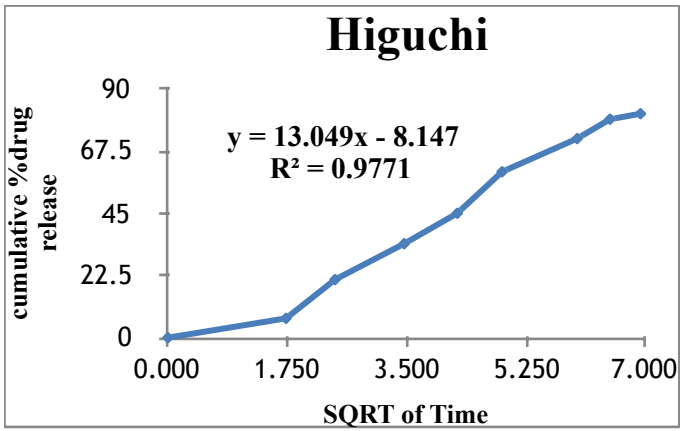


Fig. 29a: Drug release kinetic modeling graphs of batch-II









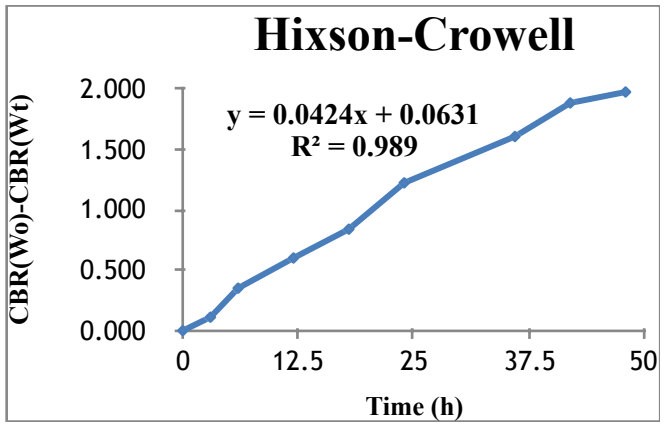


Fig. 29b: Drug release kinetic modeling graphs of batch-III

Table 22: Drug release kinetic modeling

Models	Batch-II (5-FU-NLCs)	Interpretation	Batch-III (CS-5-FU-NLCs)	Interpretation
	R^2		R^2	
Zero order	0.742	-	0.961	-
First order	0.957	-	0.957	-
Korsmeyer-Peppas	**0.978**	• Sustained release profile of 5-FU from lipid matrix • Non-Fickian diffusion	0.924	-
Higuchi model	0.947	-	0.961	-
Hixson Crowell	0.903	-	**0.990**	• Delayed release profile of 5-FU, due to chitosan swelling layer around CS-5-FU-NLCs and mucoadhesion property • Non-Fickian diffusion

*R^2 (Coefficient of regression)

6.11.2. HET-CAM model

HET-CAM assay is a good model of choice to study ocular irritation study. The results of *in vitro* ocular irritation study and irritation score (IS) of group-I (positive control, 0.1N sodium hydroxide), group-II (5-FU solution), group-III (F30, 5-FU-NLCs) and group-IV (F33, CS-5-FU-NLCs) were calculated as per Equation no. 7 and shown in Fig. 30. The results (n=3) revealed that developed formulations i.e. group-II, III and IV showed no signs of vascular injury or coagulation and found non-irritant as compared to group-I that showed severe irritation. Further, IS was calculated by observed reactions of lysis, haemorrhage, and coagulation on the CAM over a period of 6 h and net IS was calculated. The value of IS was "20.56" for group-I and for group-II, III, and IV it was "0". Hence, non-irritant nature of 5-FU was observed in group-II, III and IV with good ocular tolerance (n=3) in comparison to group-I [Bin-Jumah *et al*., 2020].

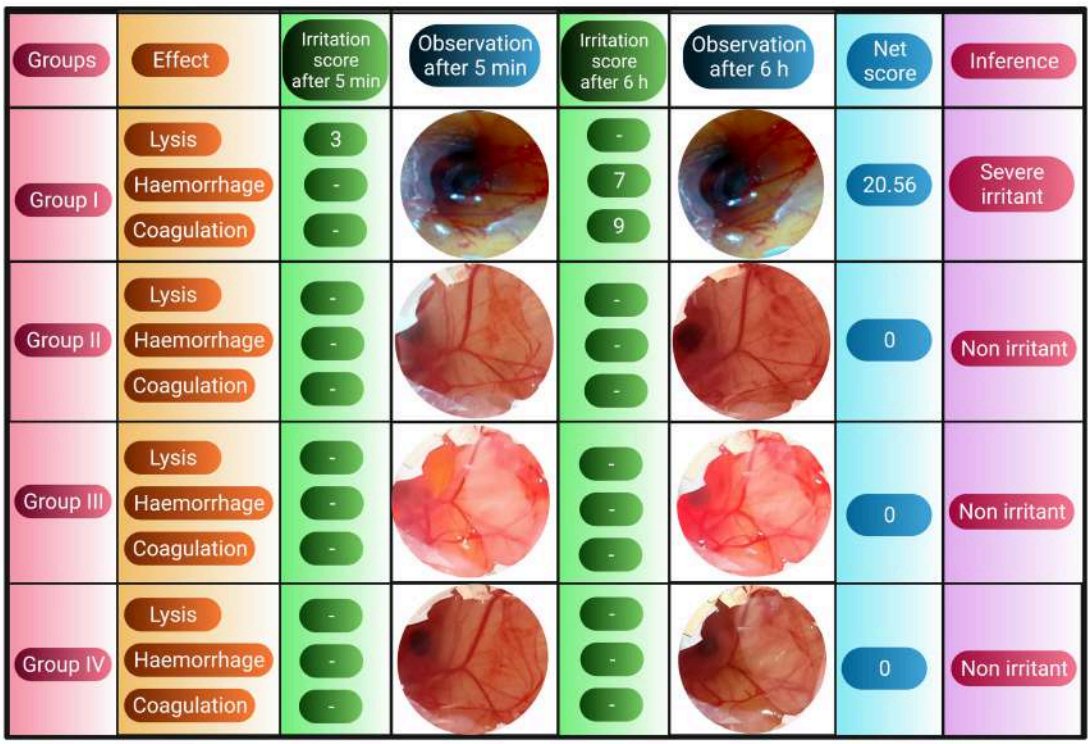


Fig. 30. Ocular irritation scores and CAM images of group I, II, III and IV

6.12. *Ex vivo p*ermeability study using goat cornea

*Ex vivo p*ermeability study was performed upto 4 h and then percentage permeation of 5-FU (± SD) was calculated (n=3) for batch-I (5-FU solution), II (5-FU-NLCs, F30), and III (CS-5-FU-NLCs, F33). After 4 h, from batch-I, II and III, around 56.20 ± 2.50 %, 71.20 ± 1.40 %, and 80.90 ± 2.20 % respectively 5-FU got permeated. The graph was plotted between percentage permeation of 5-FU vs. time and shown in Fig. 31. The results of permeation study for batch-I, II, and III were compared by one way ANOVA. The p values indicated significant differences in 5-FU permeation in case of batch-III as compared to batch-I ($p<0.01$). Moreover, in batch-III it got further improved nearly by 10 % ($p<0.05$) as compared to batch-II. Similar findings have been reported in another study, wherein it was mentioned that surface-modified form of drug loaded NLCs with CS got adhered firmly with mucin and showed improved trans corneal permeability [Chauhan *et al*., 2021].

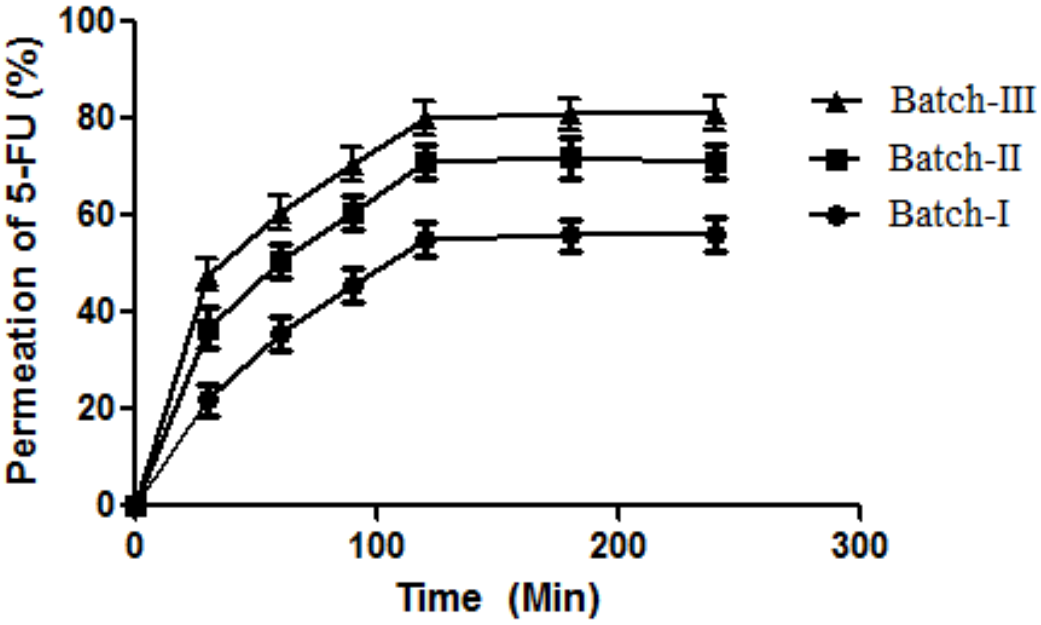


Fig. 31. *Ex vivo* permeation study of batch-I, II and III (n=3) mean ± SD

6.13. *In vivo* studies

6.13.1. CAM assay

In anti-angiogenic study, the blood vessels of eggs were arranged in a tree-like (dendritic) branching pattern with an equal distribution, covering the entire area of the CAM in case of eggs of group-I receiving positive control-sodium pyruvate) (Fig. 32). The vascular architecture of the CAM appeared to be originating from the main "λ" branch of the blood vessel i.e. central vessel of egg which further differentiated into collateral vessels viz. primary, secondary, and tertiary branches. Subsequently, increase in growth of alternative collateral vessels was seen for survival of embryos [Merckx *et al*., 2020]. As the inoculation of 5-FU solution, 5-FU-NLCs, and CS-5-FU-NLCs in group II, III, and IV eggs respectively were carried out in air cavity of chicken eggs, the immediate effects were occurring on the central vessel development. In case of group-II, III and IV eggs, central vessel's growth was largely compromised due to antiangiogenesis effect of 5-FU [Zhang *et al*., 2017]. The number of blood vessels of eggs were counted for group-I (97.00 ± 1.00), group-II (49.00 ± 2.00), group-III (44.00 ± 2.00) and group-IV (33.00 ± 1.00) as mean (± SD). The graph was plotted between mean count of blood vessels of eggs vs. groups. All groups were compared using one way ANOVA as shown in Fig. 32. The p value suggested significant reduction in mean count of blood vessels for group-II ($p<0.01$), group-III ($p<0.01$) and group-IV ($p<0.001$) as compared to group-I.

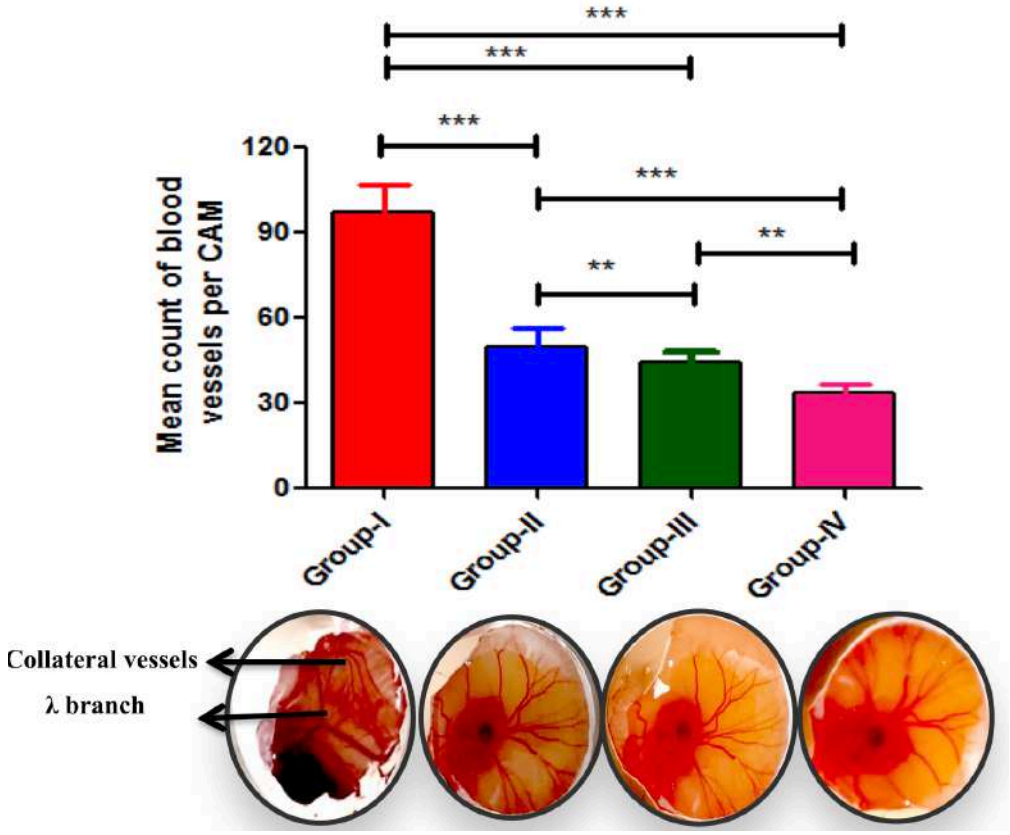


Fig. 32. Antiangiogenesis study by chorioallontoic membrane (CAM) assay: Mean ± SD blood vessels of group-I, II, III and IV (* p<0.001, ** p<0.01)**

6.13.2. DR rat model

Angiogenesis is development of new blood vessels from existing one's which may later on leads to DR in case of eyes. For prevention of DR, antiangiogenic drug plays an important role in management of DR. Different *in vivo* models have been reported to evaluate the anti-angiogenic effect of drug such as rat aortic ring assay [Go *et al*., 2003], anti-neovascularization model [Locri *et al*., 2019], DR rat model [Mahaling *et al*., 2018] etc.

Rat aortic ring assay and anti-neovascularization model are most extensively used methods to validate the anti-angiogenic effect [Go *et al*., 2003]. But, DR is not induced in these models, instead of that vascular endothelial growth factor (VEGF) was used and anti-VEGF effect of drug was determined [Sankar *et al*., 2018]. VEGF has main role in angiogenesis, which effects vascular endothelial cells, also tubulogenesis, endothelial cell proliferation, and vascular permeability [Yu *et al*., 2013]. In one of the studies, rat aorta was used to check cell proliferation, migration, and aorta tube formation after treatment with itraconazole (ITR). It has shown the absence of sprouting of vessel growth which proved anti-angiogenic effect of ITR [Selvaraj *et al*., 2019].

Another, *in vivo* anti-neovascularization model also reported for non invasive evaluation of antiangiogenenic effect. In this study, iris angiogenesis in the rat's eye was observed directly in clinical diagnostics, suggesting that animal models of iris angiogenesis could be easily evaluated and quantified *in vivo* by noninvasive methods [Locri *et al*., 2019]. In another method, corneal neovascularization was induced by alkali injury on rat eyes and anti-angiogenic effect was determined [Choi *et al*., 2017]. However, in these methods, no direct induction of DR reported.

Diabetes induced DR rat model is also reported in which single intraperitoneal injection of streptozotocin (STZ, 35 mg/kg) in PBS is injected into rats and progression of DR is observed after 3 month of DM development [Gong *et al*., 2013]. After 72 h of

the STZ injection, blood glucose levels (BGL) were monitored to ensure DM development in rats, which is further continued for next 10 weeks for successful development of DR and its associated complications including astrocyte defects, increased neuronal cell death, microglial cell activation, and microvascular leakage in rat models of T1DM [Mahaling *et al*., 2018]. At the end of the 10th week, approximately 80 µL of the respective formulations were administered as eye drops in rat's eyes once a day for 20 days. After 20 days, electroretinography (ERG) was performed to ensure DR in live rats. After this, the groups of rats were sacrificed on day 21st of treatment and isolated eye balls were analysed for histopathological examination [Seyfoddin *et al*., 2016].

The STZ induced model is better as compared to others (rat aortic ring assay and anti-neovascularization model) as it involves induction of diabetes in rats which later on leads to DR after 3 months. Hence, a real picture of DM based DR appears and anti-angiogenic effect of 5-FU was observed in rat's eyes primarily by fundus imaging and later on confirmed by histopathological examination.

6.13.2.1. Fundus imaging

Fundus images of rat's eyes were captured for group I, II, III, and IV on the 91st day (after 3 months of DM induction) and are shown in Fig. 33. These images were taken before giving any treatment to diabetes induced DR rats (to ensure development of retinopathy in rats) and after 20 days treatment given to group-II, III and IV rats. In group I rats-The retinal blood vessels were seen with an equal distribution in eye, covering the entire retinal area in group-I rats whereas after topical application of 5-FU solution, 5-FU-NLCs and CS-5-FU-NLCs formulations in the form of eye drops in right eye (RE) of group II, III and IV rats respectively for 20 days, rat retinal vessel's thickness were found to be thick. In addition, there was increase in number of retinal vessels i.e angiogenesis due to DM induced DR. The immediate effects were seen via decrease in thickness and number of retinal blood vessels i.e antiangiogenesis in group-II, III and IV rat eyes due to angiogenesis inhibitory activity of 5-FU.

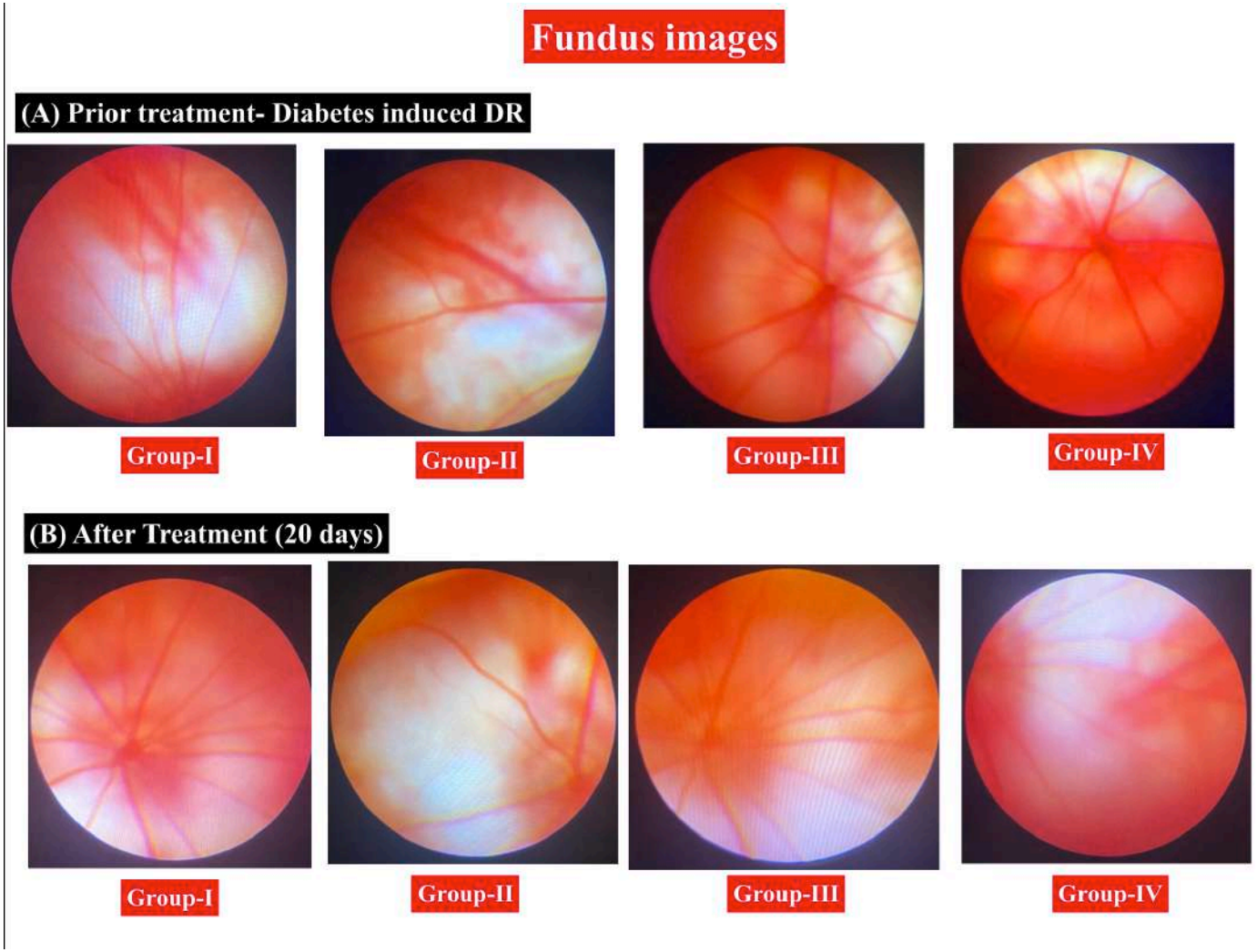


Fig. 33: Fundus images of group-I, II, III and IV

(A) Prior treatment (B) After treatment

6.13.2.2. Histopathological examination

The observations of fundus imaging was further confirmed by histopathological images captured for retina's of group I, II, III, and IV rats are shown in Fig. 34a. The retinal vessels thickness of each group was measured. The normal retinal vessel thickness was found to be 150.0 ± 2.3 mm, whereas in case of group-I, angiogenesis was observed and it was increased upto two times i.e. 300.0 ± 1.3 mm. However, decrease in thickness of retinal vessels were observed after topical application of 5-FU solution, 5-FU-NLCs and CS-5-FU-NLCs formulations in right eye of group II, III and IV rats respectively for 20 days. In group II, thickness of rat retinal vessels was found to be 220.0 ± 1.5 mm, in group III it was 180.0 ± 1.1 mm and in group IV it was 160.0 ± 2.3 mm, which indicated anti-angiogenic effect of 5-FU as shown in Fig. 34b. All results were compared using one way ANOVA. The p value suggested significant reduction in retinal vessels thickness for group-II ($p<0.01$), group-III ($p<0.01$) and group-IV ($p<0.001$) as compared to group-I.

All the obtained results indicated that CS-5-FU-NLCs offered successful delivery of 5-FU to the retina by improving its permeability at desired site.

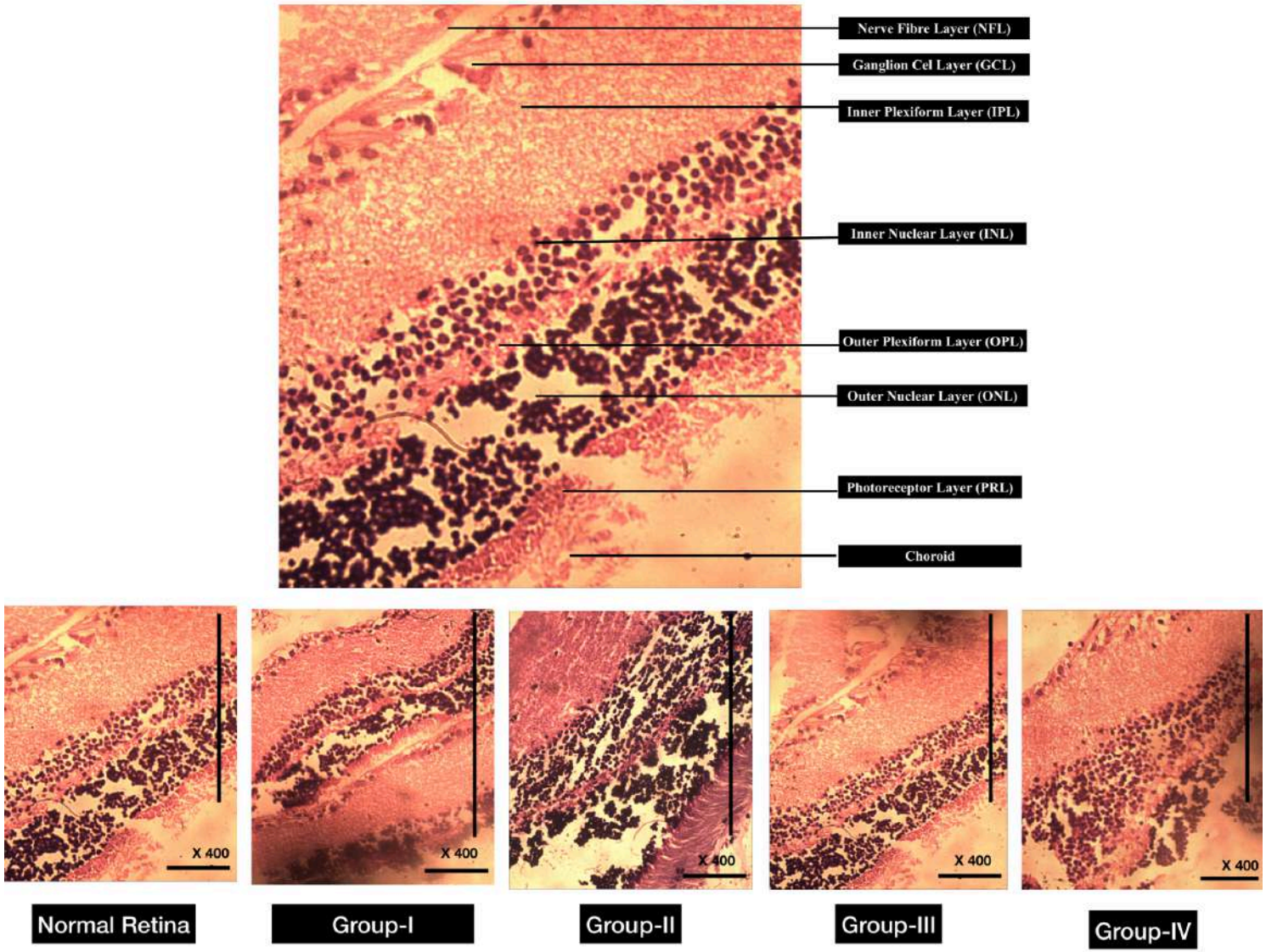


Fig. 34a. Histopathological examination of group-I, II, III and IV

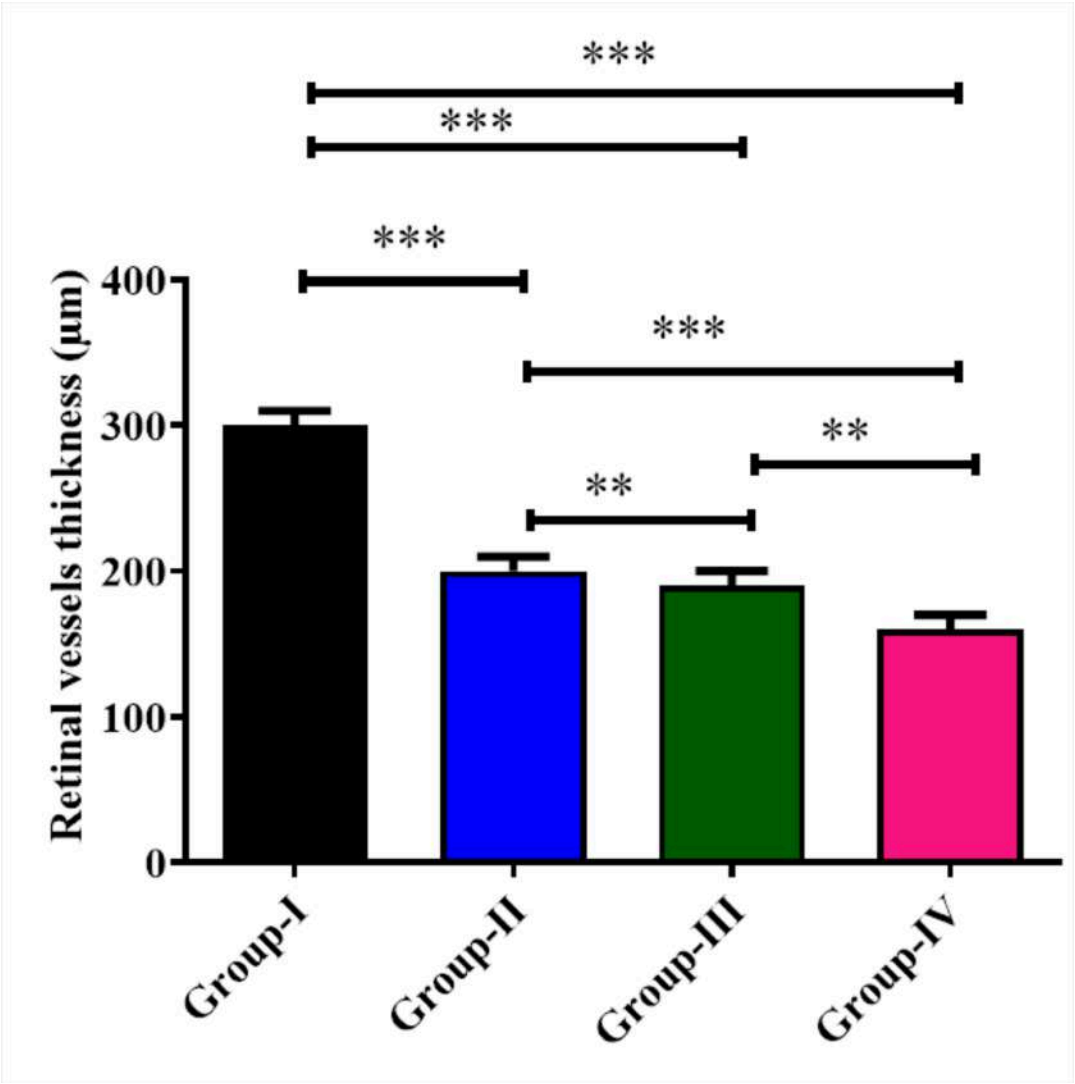


Fig. 34b. Retinal vessel thickness of group-I, II, III and IV

(* p<0.001, ** p<0.01)**

6.14. Accelerated stability studies

This study was performed using accelerated storage conditions such as temperature and relative humidity as part of the stability testing to ensure the stability of final formulation CS-5-FU-NLCs [Patil *et al*., 2016]. The CS-5-FU-NLCs was evaluated (n=3) for PS, ZP, PDI, and %EE at various time intervals (0, 1st, 3rd and 6th months). The PS (164.70 nm), PDI (0.220), and % EE (81.3 %) of CS-5-FU-NLCs (F33) were found stable, during the storage period of 6 months as evident from Table 23. There was a slight decrease in ZP (20.10 mV) upon storage. However, this decrease was non-significant as compared to fresh formulation.

Table 23: Accelerated stability studies of CS-5-FU-NLCs

Parameters	**Storage time in months**			
	0	**1st**	**3rd**	**6th**
Physical appearance	Clear and Stable	Clear and Stable	Clear and Stable	Clear and Stable
PS (nm)	163.20	164.40	164.30	164.70
ZP (mV)	21.40	20.20	20.10	20.10
PDI	0.230	0.220	0.220	0.220
% EE	81.50	81.50	81.40	81.30

7. CONCLUSION AND FUTURE PERSPECTIVES

In this research work, a successful RP-HPLC method was developed for the estimation of 5-FU and validated for linearity, range, precision, accuracy, system suitability, LOD, and LOQ as per ICH guidelines and results reflected that current method is free from interference of the impurities during the estimation of 5-FU and appropriate for estimation of 5-FU in bulk and pharmaceuticals. For development of CS-5-FU-NLCs, the excipients were selected on the basis of higher solubility of 5-FU in solid lipid, liquid lipid, surfactant, and co-surfactant. Ternary phase diagram was plotted by modified melt emulsification and ultrasonication method to develop NLCs. The 5-FU-NLCs were optimized by BBD and characterized for particle size, zeta potential, polydispersity index and % entrapment efficiency and % drug loading. To improve the permeability as well as targeting of CS-5-FU-NLCs to retina, surface modification of 5-FU-NLCs were done with 0.1 to 1.0 % w/v CS. The optimized formulation of 5-FU-NLCs and final batch of CS-5-FU-NLCs exhibited nano size range, optimum zeta potential, narrow size distribution, and higher % EE and optimum % DL. DSC thermogram and FTIR analysis shown encapsulation of 5-FU inside the matrix of lipids. SEM and TEM images revealed irregular surface of CS-5-FU-NLCs which confirmed about surface modification of 5-FU-NLCs with chitosan. *In vitro* drug release study confirmed higher drug release in CS-5-FU-NLCs as compared to 5-FU solution and 5-FU-NLCs. *In vitro* ocular irritation studies were performed by Hen's Egg Test Chorioallantoic Membrane model to ensure non-irritancy of CS-5-FU-NLCs. Results of e*x vivo* permeation study confirmed higher drug permeation in case of CS-5-FU-NLCs as compared to 5-FU solution and 5-FU-NLCs. *In vivo* ocular studies of CS-5-FU-NLCs confirmed antiangiogenic effect of 5-FU by CAM model and diabetic retinopathy induced rat model. In CAM assay, central blood vessel growth was largely compromised in eggs due to antiangiogenesis activity of 5-FU in CS-5-FU-NLCs. In diabetic retinopathy rat model, fundus images were taken to ensure DR and further examined by histopathological examination. Histopathological images were captured and normal thickness of rat’s retinal blood vessels were found to be less whereas, in

case of DR angiogenesis was observed. However, after ocular application of 5-FU solution, 5-FU-NLCs and CS-5-FUNLCs, the retinal vessel's thickness was decreased which indicated anti-angiogenic effect of CS-5-FU-NLCs as compared to 5-FU solution and 5-FU-NLCs. Accelerated stability studies for CS-5-FU-NLCs were performed upto 6 months (0, 1st, 3rd and 6th month) and CS-5-FU-NLCs were found stable, during the storage period of 6 months, but slight decrease in ZP was observed upon storage. However, this decrease was non-significant as compared to fresh formulation. All the results indicated that CS-5-FU-NLCs offered a delivery tool for targeting the 5-FU to the retina by improving its permeability at desired site.

Overall, it was concluded that the present research showed that the anticancer drug 5-FU can be utilised as an antiangiogenic agent for DR treatment. Further, this research work entailed that NLCs can be an ideal carrier for treating DR through ocular route. Moreover, the surface modification of 5-FU-NLCs using CS can offer controlled release of 5-FU, that would reduce its frequency of administration. The current treatment strategy has offered a new clinical dimension to 5-FU by repurposing it for DR as well as offered a potent non-invasive and economical treatment as compared to expensive intravitreal injections of Ranibizumab, Pegaptanib sodium, Bevacizumab and Aflibercept, which are painful, require skilled person to administer it and are expensive. The obtained results of *in vivo* studies on STZ induced DR rats showed excellent preclinical efficacy of CS-5-FU-NLCs. However, the results require good clinical correlation. However, it is pertinent to add that the lipid based nanocarriers such as NLCs are always associated with regulatory concerns related to their stability and toxicity as well as scalability. Although, the quality by design approach used in this study has helped in optimizing the best batch of NLCs with good stability and safety profile However, they need to be prepared at pilot scale and they should be tested for toxicity, stability and confirmed for absence of any significant change during scale up by comparing the results of particle size, zeta potential, drug loading and release profile with the laboratory batch. Further, the *in vivo* study results performed on rats should be also correlated with the smallest clinical batch taken for the study. Nevertheless, this

research has provided a good dimension to the formulation scientists working in ocular delivery, particular DR.

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LIST OF PATENTS, PUBLICATIONS, BOOK CHAPTER, PRESENTATIONS, CONFERENCES, AND WORKSHOP ATTENDED

Patents

- **Deep Shikha Sharma,** Sheetu, Sachin Kumar Singh, Vijay Kumar. Novel Formulations of 5-Flurouracil Against Diabetic Retinopathy And Process Thereof. Application No. IN202011044331. 2020
- **Deep Shikha Sharma**, Sheetu, Sachin Kumar Singh. A novel formulation of 5-fluorouracil for treating diabetic retinopathy. Application No. IN201911049029. 2019

Publications from PhD work

- **Deep Shikha Sharma,** Sheetu Wadhwa, Monica Gulati, Bimlesh Kumar, Nitin Chitranshi, Vivek Kumar Gupta, Mohammed Alrouji, Sharif Alhajlah, Othman AlOmeir, Sukriti Vishwas, Rubiya Khursheed, Sumant Saini, Gaurav Gupta, Flavia Zacconi, Dinesh Kumar Chellappan, Andrew Morris, Raimar Loebenberg, Kamal Dua. (2022). Chitosan modified 5-Fluorouracil nanostructured lipid carriers for treatment of diabetic retinopathy in rats: A new dimension to an anticancer drug. International Journal of Biological Macromolecules. doi: https://doi.org/10.1016/j.ijbiomac.2022.10.168 **(Impact Factor: 8.02)**
- **Deep Shikha Sharma,** Sheetu Wadhwa, Monica Gulati, Arya Kadukkattil Ramanunny, Ankit Awasthi, Sachin Kumar Singh, Rubiya Khursheed, Leander Corrie, Nitin Chitranshi, Vivek Kumar Gupta & Sukriti Vishwas. (2021). Recent advances in intraocular and novel drug delivery systems for the treatment of diabetic retinopathy. Expert Opinion on Drug Delivery, 18(5), 553-576. doi: 10.1080/17425247.2021.1846518 **(Impact Factor: 7.29)**
- **Deep Shikha Sharma**, Sachin Kumar Singh, Divya Thakur, Arya KR, Rubiya Khursheed, and Sheetu Wadhwa. (2020). Current Strategies and Future Perspective for the Effective Treatment of Diabetic Retinopathy. Current Drug Therapy, 15(4),

299-311. doi: https://doi.org/10.1080/17425247.2021.1846518**. (Impact Factor:0.80)**

- **Deep Shikha Sharma,** Sheetu Wadhwa, Sachin Kumar Singh, Arya Ramanunny, and Rajan Kumar. (2020). Estimation of 5-fluorouracil by high-performance liquid chromatography reversed-phase validated method. Research Journal of Pharmacy and Technology, 13(9), 4249. doi: https://doi.org/10.5958/0974-360X.2020.00750.7 **(Impact Factor: 1.203)**

Book chapter from PhD work

- **Deep Shikha Sharma**, Monica Gulati, Sachin Kumar Singh, Sheetu Wadhwa. (2022). Role of Novel Drug Delivery Systems in overcoming the challenges associated intraocular delivery of drugs: An overview. Multifunctional Nanocarriers Elsevier. 401-418, ISBN 9780323850414, doi: https://doi.org/10.1016/B978-0-323-85041-4.00003-2

Publications from allied work

- Rubiya Khursheed, Monica Gulati, Sheetu Wadhwa, Sukriti Vishwas, **Deep Shikha Sharma**, Leander Corrie, Aftab Alam, Sulaiman Mohammed Alnasser, Faris F.Aba Alkhayl, Zeenat Parveen, Srinivas Nammi, Dinesh Kumar Chellappan, Gaurav Gupta, Flavia Zacconi, Amie Steel, Jon Adams, Niraj Kumar Jhao, Kamal Dua, Sachin Kumar Singh (2022). Multifaceted role of synbiotics as nutraceuticals, therapeutics and carrier for drug delivery. Chemico-Biological Interactions.110223. doi: https://doi.org/10.1016/j.cbi.2022.110223. **(Impact Factor: 5.168)**
- Pooja Bhardwaj, Shailendra Kodati, **Deep Shikha Sharma,** Amit Sharma, Mangesh Pradeep Kulkarni, Sachin Kumar Singh, Vrinder Pal Singh, Gurvinder Singh, Pardeep Kumar, and Rajesh Kumar. (2021). Chewable tablets of Acacia catechu extract, an alternative to betel (paan) for mouth ulcers: formulation and *in vitro* evaluation. Current Drug Delivery, 18 (4), 500-512. doi: https://doi.org/10.2174/1567201817999200728140352. **(Impact Factor: 2.409)**

- Arya K. Ramanunny, Sheetu Wadhwa, Sachin Kumar Singh, **Deep Shikha Sharma**, Rubiya Khursheed, and Ankit Awasthi. (2020). Treatment strategies against psoriasis: principle, perspectives and practices. Current Drug Delivery, 17(1), 52-73. doi: https://doi.org/10.2174/1567201816666191120120551. **(Impact Factor: 2.409)**
- **Deep Shikha Sharma,** Amritpal Kaur, Sheetu Wadhwa, Sachin Kumar Singh, Rajesh Kumar, Rubia Khursheed, and Arya Ramanunny. (2019). Role of Egg Oil in Cosmetics: An Icing on a Cake. Research Journal of Pharmacy and Technology, 12(9), 4589-4594. **(Impact Factor: 1.203)**
- Sheetu Wadhwa, **Deep Shikha Sharma**, Meenu Mehta, Divya Thakur, Sanchit Mahajan, Sachin Kumar Singh, and Saurabh Satija.(2018). Vitamin D deficiency, skin, and sunshine: A review. International Journal of Green Pharmacy (IJGP), 12(02) . doi: https://doi.org/10.5958/0974-360X.2019.00789.3. **(Impact Factor: 0.143)**
- Souvik Mohanta, Sachin Kumar Singh, Bimlesh Kumar, Monica Gulati, Jivan Jyoti, Sananda Som, Sakshi Panchal,Indu Melkani, Mayukh Banerjee, Shubham Kumar Sinha, Rubiya Khursheed, Ankit Kumar Yadav, Vishu Verma, Rajan Kumar, **Deep Shikha Sharma**, Adil Hussain Malik, Narendra Kumar PandeySheetu Wadhwa (2018). Solidification of liquid Modified Apple Polysaccharide by its adsorption on solid porous carriers through spray drying and evaluation of its potential as binding agent for tablets. International journal of biological macromolecules, 120, 1975-1998. doi: https://doi.org/10.1016/j.ijbiomac.2018.09.181. **(Impact Factor: 4.7)**
- Kubota Mwaka Hazemba, Jivan Jyoti, Sheetu Wadhwa, Sananda Som, Souvik Mohanta, Ankit Kumar Yadav, Bimlesh Kumar, Varun Garg, **Deep Shikha Sharma,** Rubiya Khursheed, Monica Gulati, Sachin Kumar Singh (2018). “Influence of formulation parameters on dissolution rate enhancement of acyclovir using liquisolid formulation”. Asian journal of pharmaceutical and clinical research. 11 (2). doi:https://doi.org/10.22159/ajpcr.2018.v11s2.28537. **(Impact Factor: 6.5)**

- **Poster presentation**

- E-Poster presentation on the topic “Current strategies and future perspective for the effective treatment of diabetic retinopathy” on 19th April, 2019 at Chitkara College of Pharmacy, Chitkara University, Punjab
- E-Poster presentation on “Estimation of 5-Fluorouracil by High Performance Liquid Chromatography and its Validation” in 6th International conference on Pharmacy practice and clinical research and pharmacovigilance Trends-2019’ from 8-9th, November, 2019 at Chitkara College of Pharmacy, Chitkara University, Punjab

- **Oral presentation**

- Oral presentation on “Formulation Development, Characterization and *In vitro* Evaluation of 5-Fluorouracil Loaded Nanostructured Lipid Carriers” in International conference on Materials for Emerging Technologies (ICMET-21) held on 18-19th Feburary, 2022, at Lovely Professional University (LPU), Phagwara, Punjab
- Oral presentation on “ Preliminary screening of formulation variables for development of 5-Fluorouracil Loaded Nanostructured Lipid Carriers” in National conference on “Recent trends in Biomedical Sciences (RTBS-2020)” on 2-3rd July, 2021 at Lovely Professional University, Punjab
- Oral presentation on “Formulation of 5-Fluorouracil loaded nanostrructured lipid carriers for the management of diabetic retinopathy” in 14th Chandigarh Science Congress (CHASCON-2020) from 17-19th, December, 2020 at Panjab University, Chandigarh
- Oral presentation on “Estimation of 5-Fluorouracil by High Performance Liquid Chromatography and its Validation” in International Conference of Pharmacy (ICP-2019) held on 13-14th, September, 2019 at Lovely Professional University, Punjab

- **Conferences/Workshops/Webinar attended**

- Two days National Symposium on "Translational Research and Future Pharmaceutics" as a scientific committee member from 4-5th November, 2022 at JSS College of Pharmacy, Ooty.
- International conference on "Feminine Hygiene Management-Beyond Taboo" on 25-26th November, 2022 at Lovely Professional University, Punjab
- International conference of Pharmacy (ICP-2022) held on 9-11th November, 2022 at Lovely Professional University, Punjab
- International conference on Materials for Emerging Technologies (ICMET-21) held on 18-19th February, 2022, at Lovely Professional University (LPU), Phagwara, Punjab, India
- IPR Awareness Webinar on " Patenting for Academic Institutions" on 23rd December, 2021 at Lovely Professional University, Punjab
- Two days Webinar on topic "Navigating the Pathways of Research Publishing in Scopus Indexed Journals" on 24-25th September, 2021 at Research and Consultancy Cell, Vidhya Prabodhini College (VPCECM), Goa
- National Pharmacovigilance Week-2021, Theme:"Pharmacovigilance: A step towards Patient Safety" on 17-23rd September 2021 at Lovely Professional University, Punjab
- Webinar on the topic "Importance of Particle Characterization and Zeta Potential in Pharmaceutical" on 22nd July, 2021 at Anton par India Pvt Ltd, Gurgaon.
- National conference on "Recent trends in Biomedical Sciences (RTBS-2020)" on 2-3rd July at Lovely Professional University, Punjab
- Webinar on the topic "Basics of rheology for pharmaceutical applications on 11th June, 2021 at Anton par India Pvt Ltd
- Webinar on "Formulation Development of Nutraceutical / Herbal Products: Global regulation and Prospective Opportunities" on 30th April, 2021 at Lovely Professional University, Punjab
- National conference "Quality Control of Indian Medicinal Plants-Standardized raw

materials to finished products" on 25-26th March, 2021 at Maharshi Dayanand University, Rohtak

- International e-Workshop on "Computational Approaches in Drug Design & Therapeutics 20th February, 2021" at Amity Institute of Biotechnology, Amity University, Chhattisgarh
- Webinar on "Rise of Dermacosmeceutical Market as an upcoming Trend and Opportunities for Pharma Graduates" on 28th January, 2021 at Bombay college of pharmacy
- Workshop on "Intellectual property rights" on18th January, 2021 at Swami Vivekanand College of Pharmacy, Banur, & Indian Pharmacy Graduate Association Women Forum
- Short term training programme on "Computer Aided Drug Designing: A Customized and Innovative Solution to the Greatest Challenges in Chemistry" on 11-16th January, 2021 at Amar Shaheed Baba Ajit Singh Jujhar Singh Memorial College of Pharmacy, Bela (Ropar), Punjab
- International conference "Current Scenario in advancements of pharmaceutical education and research: Challenges & prospects SVCPCON-2020" on 29-31st December, 2020 at Swami Vivekanand college of pharmacy, Banur, Punjab
- Conference 14th Chandigarh Science Congress, CHASCON-2020 on 17-19th December, 2020 at Panjab University, Chandigarh
- National Virtual conference on "Pharma vision 2k25" on 27th September, 2020 supported by Association of Pharmaceutical Teachers of India (APTI)
- Webinar on"Application of Basic Statistics and Design of Experiments in Pharmaceutical Research" on 3-5th September, 2020 at Lovely Professional University, Punjab
- '6th International conference on Pharmacy practice and clinical research and pharmacovigilance Trends-2019' from 8-9th November, 2019 at Chitkara College of Pharmacy, Chitkara University, Punjab
- Conference on Integrated Association of medical, Basic and Social Scientists (IAMBSSCON 2019) on 19th October, 2019 at PGIMER, Chandigarh

- International Conference of Pharmacy (ICP-2019) held on 13-14th September, 2019 at Lovely Professional University, Punjab
- National Symposium on Innovation and entrepreneurship on 9th April, 2019 at university Institute of Pharmaceutical Sciences, Panjab University, Chandigarh.
- Conference on Pharmacy Practice Summit-2019 from 19-20th April, 2019 at Chitkara College of Pharmacy, Chitkara University, Punjab
- Conference on Pharmacy Practice Module-VII 'ETHC-2019' from18-21st April, 2019 at Chitkara College of Pharmacy, Chitkara University, Punjab
- Attended conference "AROGYA SANGOSHTHI" on 13-14th October, 2018 organized by School of Pharmaceutical Sciences, Lovely Professional University (LPU), Phagwara, Punjab, India
- Workshop on Rheometer, DLS and AFM on 3rd August, 2018 at Anton Part, Gurgaon
- Conference on the theme "Design of Experiments" at School of Pharmaceutical Sciences in association with Qsutra Technologies, Bangalore on 13-14th July, 2018 at Lovely Professional University, Punjab
- Workshop of Springer Nature on "How to Write and Publish Scientific Articles and Manuscripts" on 15th April, 2018 organized by School of Pharmaceutical Sciences, Lovely Professional University (LPU), Phagwara, Punjab, India
- Conference "PHYTOCON-2018" on "Commercializaton of Medicinal Plant Products: Lab Techniques to Trade" on 14th April, 2018 organized by School of Pharmaceutical Sciences, Lovely Professional University (LPU), Phagwara, Punjab, India
- Workshop on "Role of HPLC and Analytical Method Development in Dosage Form Design" from 2-3rd February, 2018 at Lovely Professional University, Punjab

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Chitosan modified 5-fluorouracil nanostructured lipid carriers for treatment of diabetic retinopathy in rats: A new dimension to an anticancer drug

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ABSTRACT

Diabetic retinopathy (DR) is one of the chronic complications of diabetes. It includes retinal blood vessels' damage. If untreated, it leads to loss of vision. The existing treatment strategies for DR are expensive, invasive, and need expertise during administration. Hence, there is a need to develop a non-invasive topical formulation that can penetrate deep to the posterior segment of retina and treat the damaged retinal vessels. In addition, it should also provide sustained release. In recent years, novel drug delivery systems (NDDS) have been explored for treating DR and found successful. In this study, chitosan (CS) modified 5-Fluorouracil Nanostructured Lipid Carriers (CS-5-FU-NLCs) were prepared by modified melt emulsification-ultrasonication method and optimized by Box-Behnken Design. The size, polydispersity index, zeta potential and entrapment efficiency of CS-5-FU-NLCs were 163.2 ± 2.3 nm, 0.28 ± 1.52, 21.4 ± 0.5 mV and 85.0 ± 0.2 %, respectively. The *in vitro* drug release and *ex vivo* permeation study confirmed higher and sustained drug release in CS-5-FU-NLCs as compared to 5-FU solution. HET-CAM Model ensured the non-irritant nature of CS-5-FU-NLCs. *In vivo* ocular studies of CS-5-FU-NLCs confirmed antiangiogenic effect of 5-FU by CAM model and diabetic retinopathy induced rat model, indicating successful delivery of 5-FU to the retina.

1. Introduction

Diabetic retinopathy (DR) is a common comorbidity among patients having a prolonged history of diabetes mellitus (DM). DR damages retinal blood vessels and retinal nerves. It is considered as leading cause of vision loss or complete blindness among the diabetic population worldwide [1]. Presence of anatomical and physiological retinal eye barriers poses challenge for the treatment of DR. Moreover, the blood

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retinal barriers (BRB) do not allow the movement of drugs from blood into the posterior part of the eye [2]. Consequently, the success of DR's treatment depends on targeting drug to the posterior segment of eye *i.e.* retina by crossing or by passing ocular barriers for the management of DR [3].

Ocular conventional systems include ophthalmic drops, suspensions, emulsions, and ointments that are being used for the treatment of ocular pathologies. Most of the topically applied drug entities are removed and washed off from the eyeball by mechanism of lacrimation, tear dilution, and tear turnover, ultimately causing low bioavailability of drugs. Therefore, about 5 % of topically applied drug at ocular site enters the eye [4]. The major reasons for the moderate success of existing therapies include protein binding, reduction in drug's concentration on instillation, less space at ocular site, invasive process, and high cost of treatment [5]. The intravitreal injections are also used for treating DR and found highly effective. However, these treatments are costly, painful and require invasive route and skilled person for their administration [1].

The challenges of the existing conventional dosage forms suggest to rethink towards the need for noninvasive, effective, and economical delivery systems. In recent years, novel drug delivery systems (NDDS) have emerged as effective carrier that can deliver the drug effectively to the retina for overcoming the challenges associated with conventional as well as surgical approaches [6]. Additionally, several nanocarrier have been explored to prevent diabetes related complications [7]. Different nanocarrier, for example, solid lipid nanoparticles (SLNs) [8], polymeric nanoparticles (NPs) [9,10], gold nanoparticles [2,11], nanostructured lipid carriers (NLCs) [12], liquid crystals (LC) [13], liposomes [14], and microemulsion [15] have shown several advantages as compared to conventional drug delivery systems, such as increased surface area, improved adhesion, depot formation, enhanced biocompatibility, and controlled drug release rate. The development of NDDS and their ocular application have certainly provided an edge to treat DR over existing therapies. Among them, NLCs are potential drug delivery systems for treating ocular diseases. These are generally prepared using solid as well as liquid lipids. They can be applied topically to the eyes as a non-invasive dosage form. Considering the above-mentioned advantages, nowadays NLCs are attracting more attention [16]. The present research work involves the repositioning of 5-Fluorouracil (5-FU) in the treatment of DR by loading it into NLCs.

5-FU possesses antifibrotic, anticancer, and antiangiogenic effect [17]. It is commercialized as an anticancer drug. However, due to its potent anti-angiogenic property, it can be a good candidate for treating DR. It is a Biopharmaceutical Classification System (BCS) class III drug, hence, it has good solubility but poor permeability [17]. Thus, developing a formulation that can enhance the penetration/permeability of 5-FU in the eye and make it to reach at retinal site can be a novel and non-invasive approach to treat DR [18]. The NLCs are known to overcome such challenges. Hence, an attempt has been made to develop 5-FU NLCs for the treatment of DR. 5-FU-NLCs can reach to the retina because of the presence of lipid molecules and transport the drug in a more controlled mode for the treatment of DR [19]. These NLCs can provide more availability, residence time, improved permeation of 5-FU to the targeted areas of the retina and offer non-invasive delivery *via* ocular route. Thus, they may improve patients' compliance, better ocular tolerability as well as cost effective dosage from.

NLCs have the potential to control drug release, offer higher drug loading and good bioavailability. Hence, they could be a promising drug delivery system for ocular therapy [20]. However, the instability issues and anionic nature of NLCs have hindered their ophthalmic application. The NLCs with negative charge have difficulty during their interaction with negatively charged corneal surface [21]. To overcome this problem, surface modification of NLCs with cationic polymers such as Eudragit-RS-100 [22], chitosan (CS) [12], PEG-400, stearylamine [23] have been reported. In a study reported by Niamprem et al. in 2019, it was noted that surface modification of NLCs by PEG-400, and stearylamine did not produce significant effect on particle size of drug. In another study, surface modification of lipophilic genistein with cationic polymer Eudragit-RS-100 was done. It led to interaction of genistein with corneal cells for longer duration and improved penetration. Thus, it was concluded that it can be effectively used for ocular route [22].

The use of carbohydrate based polymers such as chitosan (CS) has been used for controlling the release of drug at the target site [24]. CS has been mostly used for surface modification owing to its mucoadhesive, biocompatible, biodegradable, non-toxic, non-allergenic nature, and penetration enhancing properties [9]. It is a natural polysaccharide that consists of 2-amino-2-deoxy-h-d-glucan with glyosidic linkages. It has the ability to get adhered to negatively charged mucosal surface of eye, owing to its positive charge. Thus, resulting in electrostatic interactions which leads to prolonged residence time at drug absorption sites [25]. Also, CS has been extensively used for topical delivery of drug to the posterior side of eye segment and act as absorbefacient for ophthalmic purpose in which the positive charged groups of CS were able to interact with the cornea [25]. Furthermore, it is reported previously that surface modified NLCs can be more effective in terms of amount of drug permeated through the cornea as compared to NLCs [26,27]. Selvaraj et al. in 2019, reported that surface modification of itraconazole (ITR) loaded in NLCs using CS has been successfully used for the management of ocular neovascularization [12].

There are several studies that have been recently published wherein, CS has been used as carrier/coating material to deliver 5-FU and other anticancer drugs. For instance, Pooresmaeil et al. prepared CS microspheres, CS coated zinc-based metal-organic framework (CS/Zn-MOF) microspheres and CS coated hybrid of Zn-MOF with graphene oxide (CS/Zn-MOF@GO) microspheres of 5-FU for effective treatment of breast cancer [28]. The anticancer efficacy of developed formulations was tested on MDA-MB 231 cells. The results indicated that CS/Zn-MOF@GO ternary hybrid microspheres have provided very good anticancer efficacy with 41.2 % cell viability. Similarly, in another study, Pooresmaeil and Namazi formulated curcumin loaded pH sensitive CS microspheres. The porous MIL-88 (Fe) was prepared in the presence of the presynthesized graphene quantum dots (GQDs) (GQDs@MIL-88 (Fe)) and curcumin was loaded in it. Further, they were coated with CS. The final formulation was in the form of microsphere [CS/CUR@GQDs@MIL-88(Fe) microspheres]. The formulation showed a pH-sensitive swelling behavior and released 38.3 % of CUR at pH 5.0. Furthermore, CS/CUR@GQDs@MIL-88(Fe) exhibited better safety as compared to CUR@GQDs@MIL-88(Fe) as per their results obtained through MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) and DAPI (4′,6-diamidino-2-phenylindole) assay against MDA-MB 231 cells [29]. In another study, CS–gelatin/zinc oxide nanocomposite hydrogel scaffolds (CS–GEL/nZnO) were prepared *via in situ* synthesis of ZnO nanoparticles (nZnO). The developed scaffolds showed enhanced swelling, biodegradation, and antibacterial properties [30]. Khan et al. developed CS coated 5-FU emulsion for transdermal application. The results indicated that the developed CS coated 5-FU emulsion exhibited the good penetration profile, prevented the premature release of drugs from the nano droplets. They also enhanced permeation and the retention of the drug across the skin [31]. In another study, galactosylated NLCs were formulated for delivery of 5-FU to treat hepatocellular carcinoma. The formulation showed good anti-cancer activity against HepG(2) cell line [32]. These all studies show the potential of CS as controlled release polymer for site specific release of drugs.

As mentioned above, 5-FU is also having the antifibrotic, anticancer, and anti-angiogenic effect similar.

to that of anti-VEGF drugs. Selection of 5-FU due to it potent unutilized anti-angiogenic activity for the management of DR has been explored. However, due to its poor permeability, the use of NDDS can offer better ocular permeability. Owing to the aforementioned benefits of NLCs, these were selected as a delivery system to penetrate the drug deeper towards retina and surface modification of 5-FU-NLCs was done using CS to improve the bioadhesion. CS has the ability to adhere at the mucosal surface leading to prolonged residence time at drug absorption

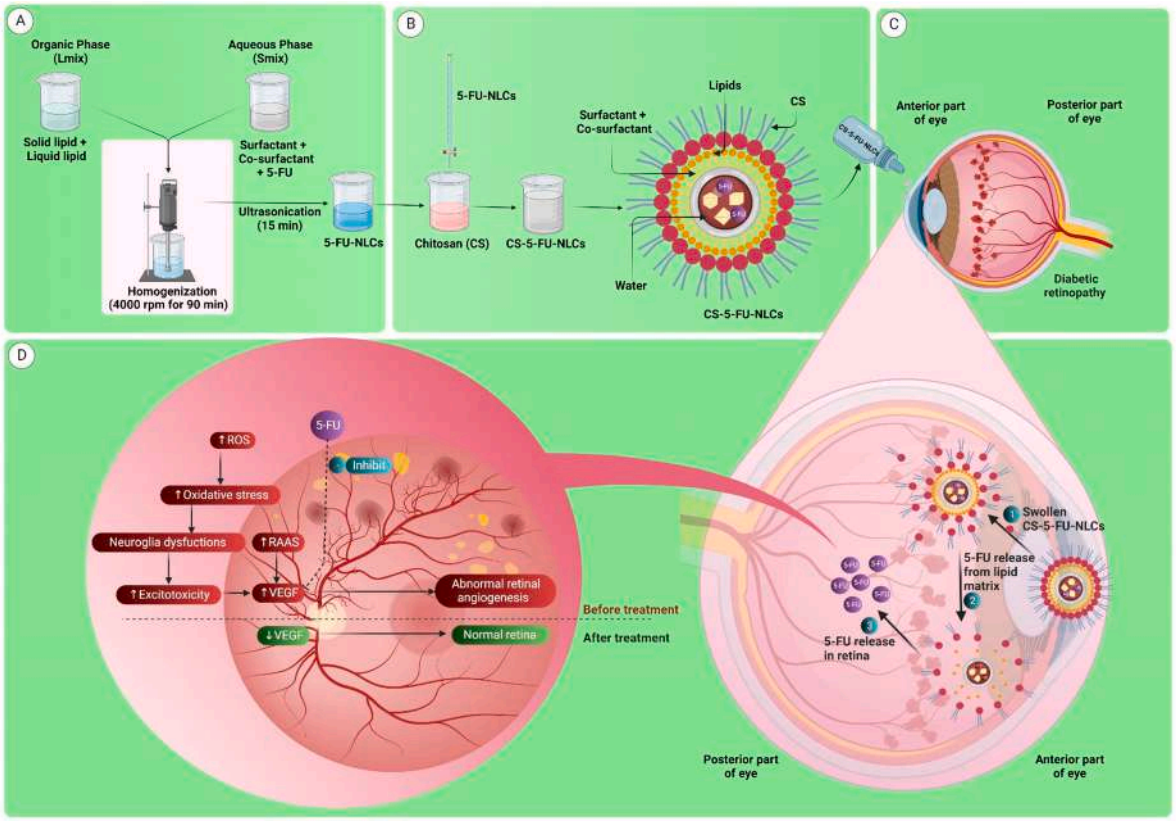


Fig. 1. Pictorial representation of a. steps involved in formulation of 5-FU-NLCs; b. coating of 5-FU-NLCs with CS (CS-5-FU-NLCs); c. topical administration of CS-5-FU-NLCs; d. mechanism involved in treating diabetic retinopathy.

sites. It is biocompatible, biodegradable, non-toxic and non-allergenic in nature. CS coated 5-FU-NLCs (CS-5-FU-NLCs) can tightly adhere to the retina of the eye because of the presence of lipid molecules. Further it has the ability to transport the drug in a more controlled fashion for the treatment and management of DR. The pictorial representation of overall strategy regarding formulation of CS-5-FU-NLCs, its topical application and mechanism involved in treating DR is shown in Fig. 1.

2. Materials and methods

2.1. Materials and instruments

2.1.1. Materials

5-Fluorouracil (5-FU) was procured as gift sample from Loba Chemie Pvt. Ltd. Mumbai, India. Propylene glycol (PG) 200, 400, and 600, span 80, polyethylene glycol (PEG) 200, 400, and 600, tween 20 and 80 were procured from Central Drug House Pvt. Ltd., New Delhi, India. All the oils (sesame oil, olive oil, cotton seed oil, groundnut oil, almond oil, soyabean oil, eucalyptus oil, mustard oil, castor oil) were procured from Global Merchants, Navi Mumbai, India. Labrafil M 2125 CS, Labrafac Lipophile WL, Labrasol ALF, and Transcutol HP were procured as gift sample by Gattefosse India Private Limited, Mumbai, India. Capmul MCM (CMCM) was gifted by Abitec Corporation, Mumbai, India. Glyceryl monostearate (GMS), analytical (HPLC) grade methanol, O-Phosphoric acid (OPA) and CS were procured from Dee Jay Corporation, Punjab, India. Double distilled water was used throughout the study. Streptozotocin (STZ) was procured from Anjan Enterprises, Amritsar, India. Double distilled water was used throughout the study.

2.1.2. Instruments

Different instruments were used throughout this study. These include, centrifuge (REMI RM-12C, Remi Electrotechnic Ltd., Varsi, Mumbai, India), differential scanning calorimeter (DSC) [DSC 6000 Perkin Elmer, USA, DSC Q200 V24.4 Build 116, Bangalore, India], electronic weighing balance (CY 360, Shimadzu Co. Ltd., Kyoto, Japan, Sansui-vibra DJ-150S-S, India), Fourier Transform Infrared Spectroscopy (FT-IR) [8400S, Perkin Elmer, India Meditech Technologies India Private Limited], Franz diffusion cell (FDC) [EMFDC-07, Meditech Technologies India Pvt. Ltd., Mumbai, India] Fundus camera (UTAS-3000 ERG system, LKC Technologies, Mumbai, India), High Performance liquid chromatography (HPLC) [HPLC LC-20AD, Shimadzu Co. Ltd., Kyoto, Japan], homogenizer (glass-teflon potter homogenizer, Thomas Scientific, USA), hot air oven (Cadmach Drying Oven, Cadmach Machinery Ltd., Ahmadabad, India), magnetic stirrer (Remi 5MLH, Vasai, Mumbai, India), microscope camera (Leica CTR 5000, Leica microsystems, Germany), melting point apparatus (Popular, India, Particle size analyzer-Malvern Zetasizer, Nano ZS90, UK), pH meter (Phan, Lab India, Mumbai, India), powder X-ray diffractometer (Bruker D8 Advance, USA), scanning electron microscope (SEM) [SEM, Joel JSM-7610F Plus, Japan], shaking water bath (Labfit, India), stability chamber (REMI CMH 10S, Remi Sales & Engineering Ltd., Mumbai, India), Transmission electron microscope (TEM) [JEM-2100 plus Electron microscope, Jeol, Japan], ultrasonication bath (UC-8120, Loba Life, Lobachemie, Mumbai, India), UV–Visible spectrophotometer (UV-1800, Shimadzu Co. Ltd., Kyoto, Japan), vortex mixer (REMI CM101, Delhi, India), 0.22 μm syringe filter (Hi Media, India).

2.2. Methodology

NLCs are drug delivery systems prepared with solid lipids as well as liquid lipids, surfactants, co-surfactants, and other agents like surface modifiers [12].

2.2.1. High-performance liquid chromatography (HPLC) method for estimation of 5-FU

A reverse phase HPLC method was developed for quantification of 5-FU in the NLCs. The method was developed on C18 (Nucleodur C18) column having 250 mm length and 4.6 mm internal diameter. The size of silica particles packed in the column was 5 μm. The mobile phase used was OPA (0.5 %) and methanol having ratio 95:5, *v*/v. Isocratic elution at a flow rate of 0.8 mL/min was carried out throughout the study. The injection volume was 20 μL. The detection wavelength was 266 nm. The drug showed its retention at 7.2 min and linearity in the range of 2–10 μg/mL with correlation coefficient of 0.992. During validation the method showed recovery of 5-FU in the range of 95 % to 100 % with <2 % relative standard deviation (RSD) among the replicated readings. This indicated excellent accuracy and precision of the method. The limit of detection and quantification for 5-FU were found to be 0.870 and 2.637, respectively [33].

2.2.2. Screening of excipients

The solubility of 5-FU in the lipids was the major concern that influences the drug entrapment efficacy (EE) and *in vitro* drug release. Various surfactants and cosurfactants (Transcutol HP, Tween 20 and 80, PG 200, 400 and 600, PEG 200, 400 and 600, Span 80), solid lipids and liquid lipids (GMS, Labrafil M 2125 CS, Labrafac Lipophile WL, Labrasol ALF, groundnut oil, soyabean oil, olive oil, cotton seed oil, mustard oil, almond oil, eucalyptus oil, castor oil, sesame oil) were selected for solubility study. In a centrifuge tube (CT), 1 mL of both solid and liquid lipids, surfactants, and cosurfactants were taken separately. An excess amount (50 mg) of 5-FU was added to each CT and mixed using vortex mixer. It was kept over water bath with continuous shaking for 24 h at 37 °C. After this, centrifugation of this mixture was done at 5000*g* for 15 min. The supernatant was filtered through membrane filter (0.22 μm). The percentage solubility of 5-FU was determined by injecting the samples to HPLC ($n = 3$) and recording their area [34,35].

2.2.3. Development of ternary phase diagram (TPD)

The results of solubility studies (as per Section 3.1) have shown high solubility of 5-FU in solid lipid (GMS), liquid lipid (Labrafil M 2125 CS), surfactant (Tween 80), and cosurfactant (Transcutol HP). These excipients were selected further to prepare NLCs by modified melt emulsification and ultrasonication method as shown in supplementary Fig. S1 [36]. By ultrasonication method, ultrasonic waves (20 kHz) provided cavitation forces to break droplets of coarse emulsion into nanoemulsion [37]. Organic phase (2 % *w/v*) was prepared by mixing L_{mix} in which different ratios of solid lipid: liquid lipid (1:2, 1:1, 2:1) were placed in a beaker on magnetic stirrer above melting point of solid lipid (70 °C). Aqueous phase (1 % w/v) was prepared by mixing S_{mix} in different ratios of surfactant: cosurfactant (1:2, 1:1, 2:1) in 50 mL of deionized water at 70 °C. This would lead to decrease in interfacial tension at higher temperature by adsorption of surfactant at oil-water interface [38], which would further result in formation of stable NLCs of smaller particle size that may lead to prevention of droplets' coalescence. Further, the L_{mix} and S_{mix} were varied from 1:9 to 9:1 ratio [39]. Using these ratios, a total of eighty-one formulations were prepared (N1 to N81) and their compositions are shown in supplementary Table S1. Molten organic phase (L_{mix}) was added dropwise (1 mL/min) to the aqueous phase (S_{mix}) with stirring at 500 rpm on magnetic stirrer to form microemulsion. After this, 50 mL of ice-cold water was added to the microemulsion with constant stirring to form NLCs. Finally, the NLCs were homogenized for 90 min at 4000 rpm and sonicated for 15 min on ultrasonic water bath. The NLCs were stored for 24 h to check their stability. The study was designed using Triplot software version 4.1.2. (Todd Thompson Software) and ternary phase diagram (TPD) was created. The regions indicating the formation of clear and transparent NLCs with absence of precipitation or phase separation were selected as NLCs. Other NLCs were rejected due to translucent appearance [39]. The results of TPD showed that among 81 prototypes prepared, N14 having L_{mix} ratio 1:1 and S_{mix} 1:2 with internal ratios of solid lipid: liquid lipid (2.5:2.5) as well as surfactant and cosurfactant (1.7:3.3), showed minimum particle size (PS), higher zeta potential (ZP), and narrow polydispersity index (PDI), which are important parameters for a good NLCs (refer Section 3.2). Hence, this formulation (N14) was used to select variables affecting responses using design of experiments [40,41].

2.2.4. Optimization of 5-FU-NLCs using Box Behnken Design (BBD)

Design of Experiment (DoE) plays an important role in optimization of various products and process parameters and have a direct impact on product quality [39]. BBD is one of the most commonly used response surface models as compared to other study designs such as Doehelrt and Central Composite Designs, as BBD possess advantages like requirement of less experimental points (three levels per factor) and high efficiency [42].

The developed formulation *i.e.* N14 (as per Section 2.2.3) was optimized by BBD for 4 factors at 3 levels *viz.* low (−1), medium (0) and high (+1) (supplementary Table S2) using Design expert software (Design Expert, Version 11.0.1, Stat-Ease Inc., Minneapolis, MN) [43]. Four factors such as solid lipids concentration (A, mg), liquid lipid concentration (B, μL), surfactant concentration (C, μL) and cosurfactant concentration (D, μL) were selected as independent variables. As per the experimental design, total 29 experimental runs were performed and accordingly, 29 prototypes (F1 to F29) were developed along with 5-FU. The 5-FU (2 % *w/v*) was dissolved in aqueous phase (S_{mix}, as per Section 2.2.2) and characterized for particle size (PS) as Y1 and percentage entrapment efficiency (% EE) as Y2. The Y1 and Y2 were chosen as response parameters [44]. The responses obtained from the 29 experimental runs (F1 to F29) were fitted to various models in statistical design [12]. Among all 29 batches (F1 to F29), F18 was found to have minimum PS (Y1) and maximum % EE (Y2) (results are shown in Section 3.3), which was further selected for validation. The optimized batch of 5-FU-NLCs (F30) was prepared and characterized for PS, ZP, PDI and % EE as discussed in Section 2.2.5.

2.2.5. Characterization of 5-FU-NLCs formulations

The complete characterization of developed formulations (F1 to F29) and optimized batch (F30) is discussed in subsequent sections.

2.2.5.1. Particle size (PS), zeta potential (ZP) and polydispersity index (PDI). The PS, ZP and PDI were measured at 25 °C by zeta sizer for all formulations (F1 to F35). Each formulation was filtered by membrane filter (0.22 μm) to remove any possible impurities and 1 mL of it was diluted in water. This was added to separate cuvette and analyzed in zeta sizer [41].

2.2.5.2. % entrapment efficiency (% EE). All developed formulations (F1 to F30) were centrifuged for 30 min at 10,000 g. The supernatant (10 mL) was collected and diluted suitably using mobile phase (methanol/O-Phosphoric acid). The amount of unentrapped 5-FU was determined by HPLC and % EE was calculated by Eq. (1) given below.

$$EE = \frac{\text{Total amount ofdrug} - \text{amount offree drug}}{\text{Totalamountofdrug}} \times 100 \quad (1)$$

2.2.6. Surface modification of 5-FU-NLCs with chitosan (CS)

From the above parameters used for characterization (as discussed in Section 2.2.5), optimized 5-FU-NLCs formulation (F30) was validated and selected on the basis of minimum PS and maximum % EE (results are shown in Section 3.4). Hence, this optimized batch (F30) was selected for further surface modification with CS using its different amount to form CS-5-FU-NLCs (F31 to F35) [14]. The amounts of CS added to NLCs batches F31 to F35 (100 mL each) were 100 mg (0.1 % *w/v*), 200 mg (0.2 % w/v), 400 mg (0.4 % w/v), 600 mg (0.6 % w/v) and 800 mg (0.8 % w/v), respectively. Acetic acid (0.1 % *v*/v) was used to dissolve CS in order to increase cross linking potential. After this, F30 batch was added

drop wise to beaker containing CS with continuous stirring (500 rpm) on magnetic stirrer for 30 min [12,45]. These batches (F31 to F35) were kept undisturbed overnight for cross linking of CS with 5-FU-NLCs which led to complete surface modification of 5-FU-NLCs with CS.

2.2.7. Characterization of CS-5-FU-NLCs formulations

2.2.7.1. Particle size (PS), zeta potential (ZP) and polydispersity index (PDI). The PS, ZP and PDI for developed formulations (F31 to F35) were measured by zeta sizer as per procedure earlier mentioned in Section 2.2.5.1 [41,46].

2.2.7.2. % entrapment efficiency (% EE). The % EE for developed surface modified NLCs (F31 to F35) were determined ($n = 3$) as per procedure described in Section 2.2.5.2 [33].

On the basis of minimum PS and maximum % EE (results are shown in section 3.5), F33 (CS-5-FU-NLCs) formulation was selected for further studies.

2.2.8. Fourier transform infrared spectroscopy (FTIR)

In order to find characteristic functional groups of 5-FU, 5-FU-NLCs (F30), CS-5-FU-NLCs (F33) and CS as well to confirm surface modification by coating of CS over NLCs, FTIR spectroscopy was carried out by using FTIR (Perkin Elmer, India Meditech Technologies India Pvt. Ltd., 8400S). The sample (5 mg) was taken and mixed with KBr in the ration of 1:3 and pressed using hydraulic press. A thin disc was prepared and subjected for spectral analysis. Scan was taken at wavelength 4000 cm^{-1} to 400 cm^{-1} [47,48].

2.2.9. Differential scanning calorimetry (DSC)

DSC is a thermo analytical technique in which the difference between the amount of heat required to increase the temperature of sample and the reference sample is measured as a function of time and temperature. It is used extensively to determine the melting point, glass transition temperature, purity, and thermal decomposition of drug. The DSC thermograms for pure 5-FU, CS, optimized formulation (F30) and surface modified formulation (F33) with CS were recorded. In this, 2.5 mg of sample was heated in a pierced aluminium pan from 0 to 350 °C at a heating rate of 10 °C/min under a steam of nitrogen at a flow rate of 50 mL/min. The thermograms were recorded and further analyzed [42].

2.2.10. Scanning electron microscopy (SEM)

The surface morphology of developed formulations (F30 and F33) was examined by FE-SEM. It produced images of a sample surface by scanning the surface coated with gold under a focused beam of electrons at a scale of 100 nm [42]. A thin film of formulation was prepared and coated with gold. Further it was subjected to the sample cavity of SEM and analyzed.

2.2.11. Transmission electron microscopy (TEM)

TEM was performed to analyze morphological changes in developed formulations (F30 and F33). The sample was prepared by staining one drop of each of F30 and F33 formulation using 1 % aqueous solution of phosphotungstic acid having negative charge. These were placed into pioloform-coated copper grid (200 μm) using a micropipette. This thin film was left 1 h for air drying. This was analyzed under the TEM at 50–80 kV at a scale of 500 nm [49].

2.2.12. Robustness to pH change

It is important to note that change in pH sometimes affects the size and zeta potential of formulations. Hence, the stability of formulations should be checked in medium having different pH. Hence, the size and zeta potential analysis of CS-5-FU-NLCs was also carried out in three different medium having pH 1.2 (0.1 N HCl), pH 4.5 (acetate buffer), and pH 7.4 (phosphate buffer) [50]. The size and zeta potential were measured by zeta sizer as per procedure earlier mentioned in Section 2.2.5.1.

2.2.13. In vitro studies

2.2.13.1. Drug release and kinetic study. The drug release studies of developed formulations (F30 and F33) were performed using dialysis bag technique and results were compared with 5-FU solution [12]. Prior to experimentation, dialysis membrane (molecular weight cut off −12,000–14,000 Da and pore size 2.4 nm) was washed with distilled water to remove sulphate ions. Afterwards, these were soaked in buffer (pH 7.4) overnight for activation [51]. The NLCs (2 mL) containing 5-FU equivalent to 0.2 mg/mL from each of batch I (5-FU solution), batch II (5-FU-NLCs, F30) and batch III (CS-5-FU-NLCs, F33) was added into dialysis bag and sealed properly. Each bag was placed in separate 250 mL beaker having 100 mL buffer (pH 7.4) as release medium at 37 ± 0.5 °C on magnetic stirrer (100 rpm). It is important to note that the objective of our study was to check the release of 5-FU in the ocular fluids, whose pH is usually found to be 7.4 [50,52]. Hence, the release was exclusively checked at that pH. The sample (1 mL) was taken at different time intervals *i.e.* 0, 3, 6, 12, 18, 24, 36, 42 and 48 h and the same volume of fresh buffer was replaced for maintenance of sink conditions. The cumulative percentage drug release of all the samples was analyzed by injecting them to HPLC ($n = 3$) and recording their area with respect to time [34]. The graph was plotted between % cumulative drug release *vs.* time. To understand the release kinetics of formulation, the release data was fitted into various kinetic models *viz.* zero order, Korsmeyer-Peppas, first order, Higuchi and Hixson-Crowell for F30 and F33 formulation [53,54].

2.2.13.2. Hen's egg test using chorioallantoic membrane (HET-CAM) model. For ocular irritation study, white leghorn chicken eggs were purchased from M/s Sahota Hatchery Centre, Jalandhar, India and incubated for 10 days at 37 ± 0.5 °C temperature and 75 ± 5 % Relative Humidity (RH). On the 10th day, the eggs ($n = 3$) were candled to check viability of the embryos. The inner membrane was removed carefully to reveal the CAM and eggs were divided into four groups (n = 3). All the groups were treated as described here: group I received 0.5 mL of positive control (0.1 N NaOH); group II received 0.5 mL of 5-FU solution (0.2 mg/mL) in water; group III received 0.5 mL of F30 (0.2 mg/mL), and group IV received 0.5 mL of F33 (0.2 mg/mL). NLCs were applied directly to the CAM using a micropipette. After treatment of CAM with group - I to IV formulations, the membrane was checked for possible vascular changes such as haemolysis, lysis, and coagulation. The reactions were observed after 5 min and end of 6 h. Then ocular irritation score (IS, irritation score of 0–0.9 indicated non irritant, 1–4.9 indicated slightly irritant, 5–8.9 indicated moderate irritant, 9–21 indicated irritant) was calculated using the following Eq. (2) [12,55].

$$IS = \frac{301 - \text{Haemolysis}}{300} \times 5 + \frac{301 - \text{Lysis}}{300} \times 7 + \frac{301 - \text{Coagulation}}{300} \times 9 \quad (2)$$

2.2.14. Ex vivo permeability study using goat cornea

The procurement of goat eyeballs was done from M/s Sagar Slaughter House, Bhagat Singh Chowk, Jalandhar City (Regd. No.-22,121,662,000,152) and transported to laboratory under cold (4 °C) saline conditions. The cornea (5–6 mm) was removed and washed with cold saline was done. This study was conducted in FDC and corneal side was continuously kept in an intimate contact with developed formulations. In batch I, 1.0 mL of 5-FU solution (0.2 mg/mL) in water, batch II, 1.0 mL of F30 (0.2 mg/mL), and batch III, 1.0 mL of F33 (0.2 mg/mL) formulations were taken in donor compartment. The receptor compartment contained simulated tear fluid (STF) of pH 7.4. FDC was kept over magnetic stirrer at 100 rpm and temperature of buffer was maintained at 37 ± 0.5 °C. Samples (2 mL) were taken at different time intervals *i.e.*, 30, 60, 90, 120, 180 and 240 min and the same volume of

fresh buffer was replaced for maintenance of sink conditions. The aliquots were analyzed by HPLC ($n = 3$) and permeation of 5-FU was calculated [34,56].

2.2.15. In vivo studies

2.2.15.1. Anti-angiogenesis study using CAM assay. The *in vivo* CAM assay was performed on white leghorn chicken eggs (4 days old) at M/s Sahota Hatchery Centre, Jalandhar. All the eggs (n = 3) were cleaned with 70 % alcohol prior to incubation. These eggs were incubated at 37 ± 0.5 °C and at 75 ± 5 % RH. Afterwards, the eggs were divided into four groups (n = 3). On the 5th day, the upper surface of all eggs was pierced with needle and group I was injected with 10 µL of standard positive control (10 µL pyruvic acid), group II was injected with 10 µL 5-FU solution (0.2 mg/mL) in water, group III was injected with 10 µL F30 (0.2 mg/mL), and group IV was injected with 10 µL F33 (0.2 mg/mL). The formulations were injected into the cavity *via* opening. *Re*-incubation of all eggs was done after sealing of openings with adhesive tape. On the 12th day, the CAM membrane was examined in order to check them for any sign of angiogenesis [57].

2.2.15.2. In vivo studies in a diabetic retinopathy rat model. Prior to conduct of experiment, animal approval (protocol number LPU/IAEC/2021/81) was received for ethical conduct of experiment by Institutional Animal Ethics Committee (IAEC) of School of Pharmaceutical Sciences, Lovely Professional University. The Sprague Dawley male rats (90 days old) with average body weight of 230 ± 14 g were purchased and housed at 22 ± 2 °C temperature, 50 % relative humidity (R.H.) and 12 h light/dark cycle in animal house. The rats were divided equally into 4 groups ($n = 8$). The control group (Group I) of rats received 0.1 M PBS (Phosphate Buffer Saline, pH 7.4), the experimental groups (Group II, III and IV) received injection of streptozotocin (STZ, 35 mg/kg) in PBS *via* intraperitoneal route. After 72 h of the STZ injection, blood glucose levels (BGL) were monitored to ensure development of diabetes in rats. Both control and diabetic rats were fed with normal diet. Body weight and BGL of each rat was checked at the end of every week for a period of 10 weeks. The ten week period was chosen because it was reported for successful development of DR and its associated complications including astrocyte defects, increased neuronal cell death, microglial cell activation, microvascular leakage in rat models of T1DM [58]. At the end of the10th week, in Group-I rats, left eye (LE) and right eye (RE) were administered with PBS as eye drops, in group-II rats, LE was administered with PBS, RE was administered with 5-FU solution (0.2 mg/mL)] in water, in group-III rats, LE was administered with placebo NLCs, RE was administered with F30 (0.2 mg/mL), in group-IV rats, LE was administered with placebo NLCs, RE was administered with F33 (0.2 mg/mL) formulation. Approximately 80 µL of the respective eye drops were administered once a day at fixed time for 20 days. After 20 days, fundus imaging was performed to ensure presence of DR in live rats. After confirmation of DR, rats were sacrificed on day 21 and eye balls were isolated. These were analyzed for histopathological examination [56].

2.2.15.2.1. Fundus imaging. All rats were kept in a dark room for 16 h (dark adaptation) and atropine sulphate eye drops were administered in each rat's eye. The funds imaging was carried out using fundus camera (Fundus camera-UTAS-3000 ERG system, LKC Technologies, Mumbai, India) [59,60].

2.2.15.2.2. Histopathological examination. The excised eyeballs were immediately placed in 4 % *v*/v paraformaldehyde (PFA). Fixation of the eye balls was done by enucleation and cutting into two hemispheres to remove the vitreous humor and lens. Then, the tissues were embedded in paraffin wax. Their tissue sections were prepared using a microtome. The sections were deparaffinized and hydrated in a gradient of ethanol solutions (100, 95, and 70 % ethanol) and stained with hematoxylin and eosin (H&E). The images were captured using a microscope [61].

2.2.16. Accelerated stability studies

The CS-5-FU-NLCs formulation (F33) was kept in a stability chamber for 6 months at 40 ± 0.2 °C and 75 ± 5 % RH. The results were evaluated ($n = 3$) in terms of PS, ZP, PDI and % EE at various time intervals such as, 0, 1st, 3rd, and 6th month [62].

2.2.17. Statistical analysis

All the experimental data (n = 3) were expressed as mean ± standard deviation (SD). Statistical assessment of the developed data was accomplished by one way ANOVA using GraphPad Prism version 7.0 (GraphPad Software Inc., CA, USA) [63] and their results were compared with the results of fresh formulation.

3. Results and discussion

3.1. Screening of excipients

The screening of excipients was done on the basis of solubility studies. The results are shown in Fig. 2. Among selected surfactants and co-surfactants, the solubility of 5-FU was found decreasing in the following order: Transcutol HP (97.20 %) > Tween 80 (95.20 %) > Propylene glycol 200 (93.98 %) > Tween 20 (93.40 %) > PEG 200

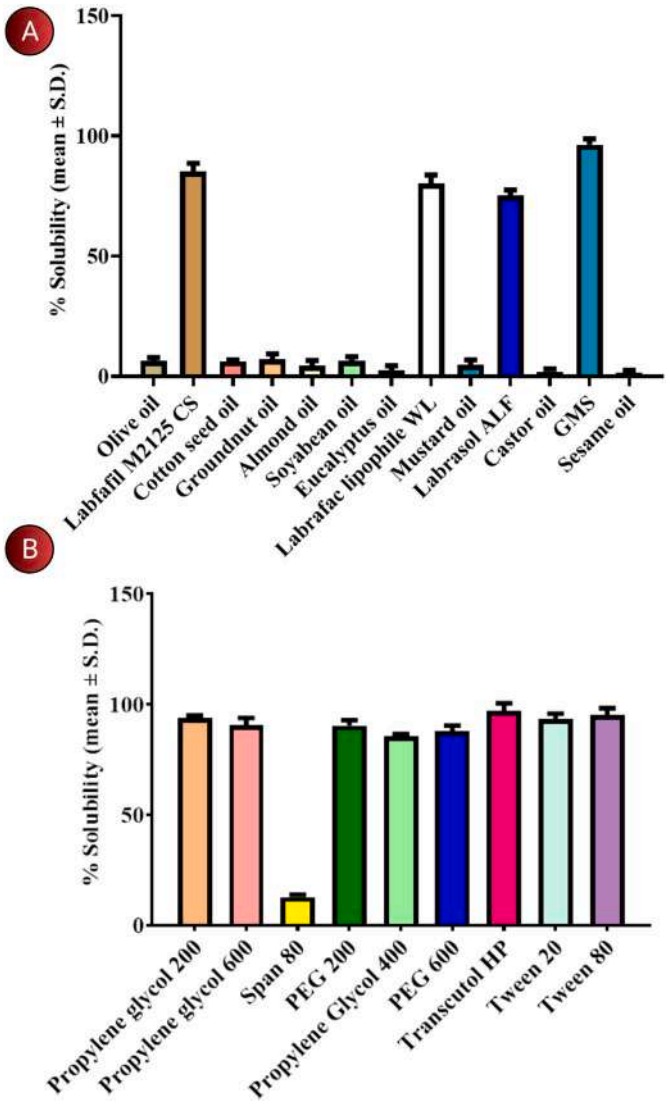


Fig. 2. Solubility of 5-FU (A) Solid lipids and Liquid Lipids (B) Surfactants and co-surfactants.

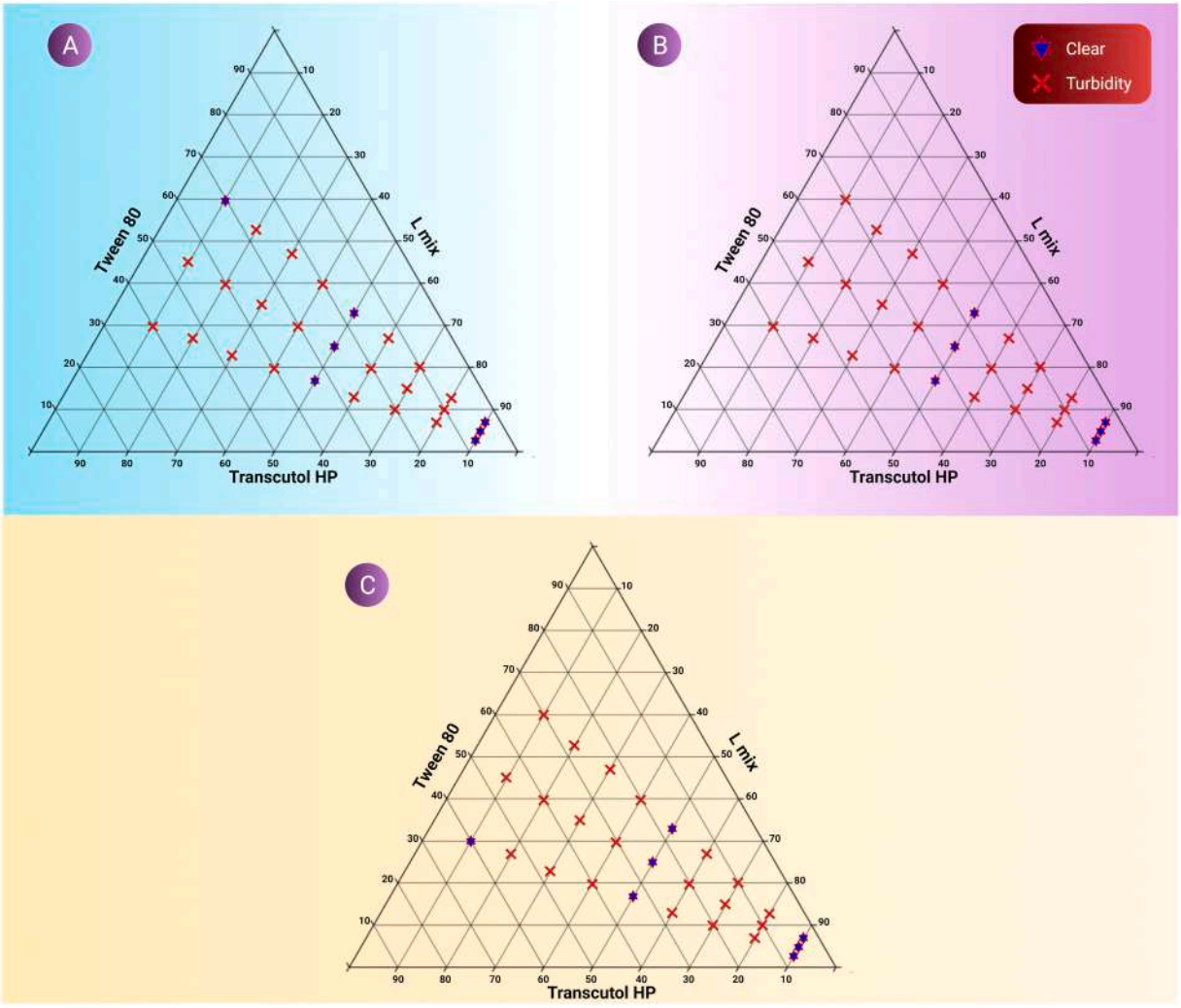


Fig. 3. Ternary phase diagram for 81 NLCs prototypes (A.) L_{mix} 1:1 (N1-N27), (B.) L_{mix} 1:2 (N28-N54), (C.) L_{mix} 2:1 (N55-N81).

(90.38 %) > PEG 600 (88.00 %) > Propylene glycol 400 (85.50 %) > Propylene glycol 600 (40.81 %) > Span 80 (12.75 %).

The solubility of 5-FU in various solid and liquid lipids was found decreasing in the following order:

GMS (96.31 %) > Labrafil M 2125 CS (85.31 %) > Labrafac Lipophile WL (80.31 %) > Labrasol ALF (75.31 %) > Groundnut oil (7.05 %) > Soyabean oil (6.52 %) > Olive oil (6.36 %) > Cotton seed oil (6.31 %) > Mustard oil (4.76 %) > Almond oil (4.45 %) > Eucalyptus oil (2.33 %) > Castor oil (1.98 %) > Sesame oil (1.40 %).

Highest solubility of 5-FU was observed in GMS (solid lipid), Labrafil M 2125 CS (liquid lipid), Tween 80 (surfactant), and Transcutol HP (cosurfactant). GMS produced solvation effect due to presence of its long chain fatty acids, which resulted in its higher penetration into the surfactant chain's layer. This caused improvement in the rigidity of the interface [64,65]. Labrafil M 2125 CS increased the solubility of 5-FU in NLCs which may resulted in improvement of availability of drug at desired site. In addition, lipid-based formulations are reported to maintain suitable nanometer size range and narrow size distribution, independently of the experimental conditions [66]. Tween 80 is reported to decrease the interfacial tension between lipids and water to form protective coating around droplets of NLCs which helps in prevention of coalescence of particles. It is pertinent to mention here that the critical micellar concentration (CMC) of Tween 80 is 0.012–0.015 mM [67] and its concentration used in the present work was above the CMC to ensure optimum particle size (PS), high drug solubilization, drug loading, and long-term stability. Transcutol HP acts as permeation enhancer as well as helps in increasing the solubility of drugs such as 5-FU [68].

Owing to the above mentioned advantages, GMS (solid lipid), Labrafil M 2125 CS (liquid lipid), Tween 80 (surfactant), and Transcutol HP (cosurfactant) were further selected for development of 5-FU-NLCs.

3.2. Construction of ternary phase diagram (TPD)

TPD of trial batches (N1 to N81) were constructed and nano-region of TPD was labelled on basis of clear NLCs formed and shown in Fig. 3. The results of TPD showed that few ratios of L_{mix} and S_{mix} shown clear NLCs with no phase separation (N5, N9, N14, N18, N23, N27, N32, N36, N41, N45, N50, N54, N59, N63, N68, N72, N77, N81). It was pertinent to add here that clear NLCs were formed when L_{mix} and S_{mix} were mixed in varying ratios (as per Section 2.2.3). Among various NLCs prototypes wherein ratio of L_{mix} was 1:1 and S_{mix} was 1:1, 1:2, 2:1, only six prototypes (N5, N9, N14, N18, N23, N27, as shown in Fig. 3.A) have shown clear NLCs. Same results were obtained for L_{mix} having ratio 1:2, and S_{mix} 1:1, 1:2, 2:1 (N32, N36, N41, N45, N50, N54 as shown in Fig. 3. B). In case where the ratio of L_{mix} was 2:1 and S_{mix} was 1:1, 1:2, and 2:1, total six NLCs formulations (N59, N63, N68, N72, N77, N81 as depicted in Fig. 3.C) were found to be clear. From TPD, it was also observed that different ratios of solid and liquid lipid have significant impact on clarity of NLCs. This indicated that solid and liquid lipid had more impact on PS

Table 1
BBD based experiments showing effect of independent variables affecting responses related to 5-FU-NLCs.

Run	Factor 1 A: GMS (mg)	Factor 2 B:Labrafil M 2125 CS (μL)	Factor 3C: Tween 80 (μL)	Factor 4 D: Transcutol HP (μL)	Response 1: Particle size nm (Y1)	Response 2: Zeta potential (mV) (Y2)	Response 3: PDI (Y3)	Response 4: % EE (Y4)
F1	3.5	450	200	50	146.0	−19.6	0.42	72.8
F2	4.5	350	200	350	146.9	−20.1	0.50	80.5
F3	3.5	350	300	350	143.9	−17.4	0.38	81.0
F4	3.5	350	200	200	155.0	−18.0	0.34	75.4
F5	2.5	450	200	200	143.9	−17.4	0.43	80.0
F6	3.5	350	200	200	155.0	−18.3	0.24	73.2
F7	4.5	450	200	200	160.4	−17.1	0.42	74.9
F8	2.5	350	200	50	143.9	−18.0	0.43	81.0
F9	3.5	350	200	200	155.0	−19.3	0.26	76.0
F10	3.5	250	300	200	148.0	−15.0	0.36	78.0
F11	3.5	350	200	50	146.0	−16.0	0.26	78.0
F12	3.5	450	300	200	155.0	−17.4	0.43	76.0
F13	3.5	350	200	200	155.0	−19.0	0.37	70.2
F14	4.5	350	300	200	145.0	−19.4	0.41	78.3
F15	3.5	350	300	50	147.0	−20.4	0.41	75.4
F16	2.5	350	200	350	141.4	−19.6	0.42	79.4
F17	3.5	450	200	350	144.9	−15.0	0.44	82.3
F18	3.5	350	100	50	129.4	−17.0	0.21	81.0
F19	3.5	350	100	350	144.4	−19.4	0.45	77.0
F20	3.5	450	100	200	142.0	−15.0	0.24	80.0
F21	2.5	250	200	200	161.0	−17.1	0.42	75.4
F22	2.5	350	300	200	156.0	−16.6	0.37	71.6
F23	4.5	350	100	200	143.9	−17.4	0.34	77.0
F24	3.5	350	200	200	148.7	−20.5	0.36	75.5
F25	2.5	350	100	200	146.2	−17.0	0.26	78.8
F26	3.5	250	100	200	156.0	−14.0	0.37	71.6
F27	4.5	250	200	200	140.4	−13.8	0.41	80.6
F28	3.5	250	200	350	155.0	−14.0	0.41	75.4
F29	4.5	350	200	50	142.2	−17.8	0.32	78.9

than surfactants and co-surfactants [69].

Out of all the 81 NLCs prototypes, only clear NLCs were selected and characterized for PS, ZP and PDI (supplementary Table S3). Their PS was ranged from 110.20 ± 3.65 nm (N14) to 251.40 ± 3.85 (N63). ZP was ranged from −9.44 ± 1.25 mV (N32) to −27.40 ± 2.36 mV (N54). PDI was ranged from 0.24 ± 0.03 (N14) to 0.45 ± 0.16 (N10). The results indicated that higher amount of liquid and solid lipid played a significant role in decreasing the PS of NLCs. Moreover, all these formulations were found stable after 48 h at 40 °C. On the basis of minimum PS, clarity and stability NLCs, N14 was selected further for preparing 5-FU-NLCs.

3.3. Screening and optimization of formulation variables

DoE (Design of experiments) tools (lack of fit test, sequential model sum of squares, and model summary statistics) were used for carrying out the statistical analysis of the model used. The lower value of standard deviation (S.D.), lower predicted residual error sum of square, high R^2 (coefficient of determination) and $p < 0.0001$ suggested selection of quadratic model for responses Y1 (PS) and Y2 (% EE). In all the cases, $p < 0.05$ indicated that the model was significant and valid. The Analysis of Variance (ANOVA) was used for determining significance and magnitude of independent variables as well as adequacy of model which revealed that the responses have responded significantly to the independent variables. The observed responses of 29 batches are shown in Table 1. The value of R^2 was found >0.500 for all the responses. The difference in the values of adjusted and predicted R^2 for responses Y1 and Y2 was <0.2, indicating their adequacy to the independent variables. The results obtained for the lack of fit test were found to be non-significant for responses Y1 and Y2. The results for ANOVA are summarized in supplementary Table S4. Moreover, variance inflation factor (VIF) for responses Y1 and Y2 were found <1.5, which measures about the inflation in the variances of the parameter and estimates the multicollinearity potential. The obtained polynomial equations for responses Y1 and Y2, are given in Eqs. (3) and (4). In this equation, positive signs indicated synergistic effect on the responses while the negative signs specified antagonistic effect on responses by factors.

$$Y1 = +153.93 - 1.13A - 0.8373B + 2.75C + 2.02D + 9.27AB - 2.18AC + 1.80AD + 5.25BC - 3.56BD - 4.52CD - 2.46A^2 + 0.443B^2 - 4.26C^2 + 7.77D^2 \quad (3)$$

$$Y2 = +74.12 + 0.3333A + 0.2046B - 0.425C + 0.2766D + -2.57AB + 2.13AC + 0.8000AD - 2.6BC + 4.16BD + 2.40CD + 2.08A^2 + 1.22B^2 + 0.7439C^2 + 3.52D^2 \quad (4)$$

These polynomial equations further helped in generating 2D and 3D response surface plots which showed same effects.

3.3.1. Particle size (PS)

The perturbation plot (Fig. 4.A.), 2D contour plots (Supplementary Fig. S2.A) and 3D response surface plots (Fig. 5.A.) for PS were plotted. In perturbation plot, it was observed that PS was highly influenced by factor D. The 2D and 3D plots revealed that response Y1 (PS) was directly influenced by factors A (solid lipid concentration) and B (liquid lipid concentration) while C (surfactant concentration) and D factor (cosurfactant concentration) have indirect effect. To check the relationship between excipients and PS, NLCs with different concentration of solid and liquid lipid, surfactant, and cosurfactant were prepared and their PS was measured. During formulation, while decreasing the concentration of solid lipid from 4.5 mg to 3.5 mg and liquid lipid from 450 μL to 350 μL, the viscosity of NLCs also decreased from 10 cP to 4 cP, which lead to decrease in the surface tension, thus PS got decreased [70]. Further, decrease in concentration of Tween 80 from 300 μL to 100 μL and Transcutol HP from 350 μL to 50 μL caused decrease in the PS. This was due to the availability of more amount of surfactant that completely covered the oil droplet. The surfactant concentration used in this study was above the CMC to ensure high drug solubilization and loading, optimum PS, and long-term stability [71]. The maximum PS was observed in F21 (161.0 nm) and minimum PS was noted for F18 (129.4 nm).

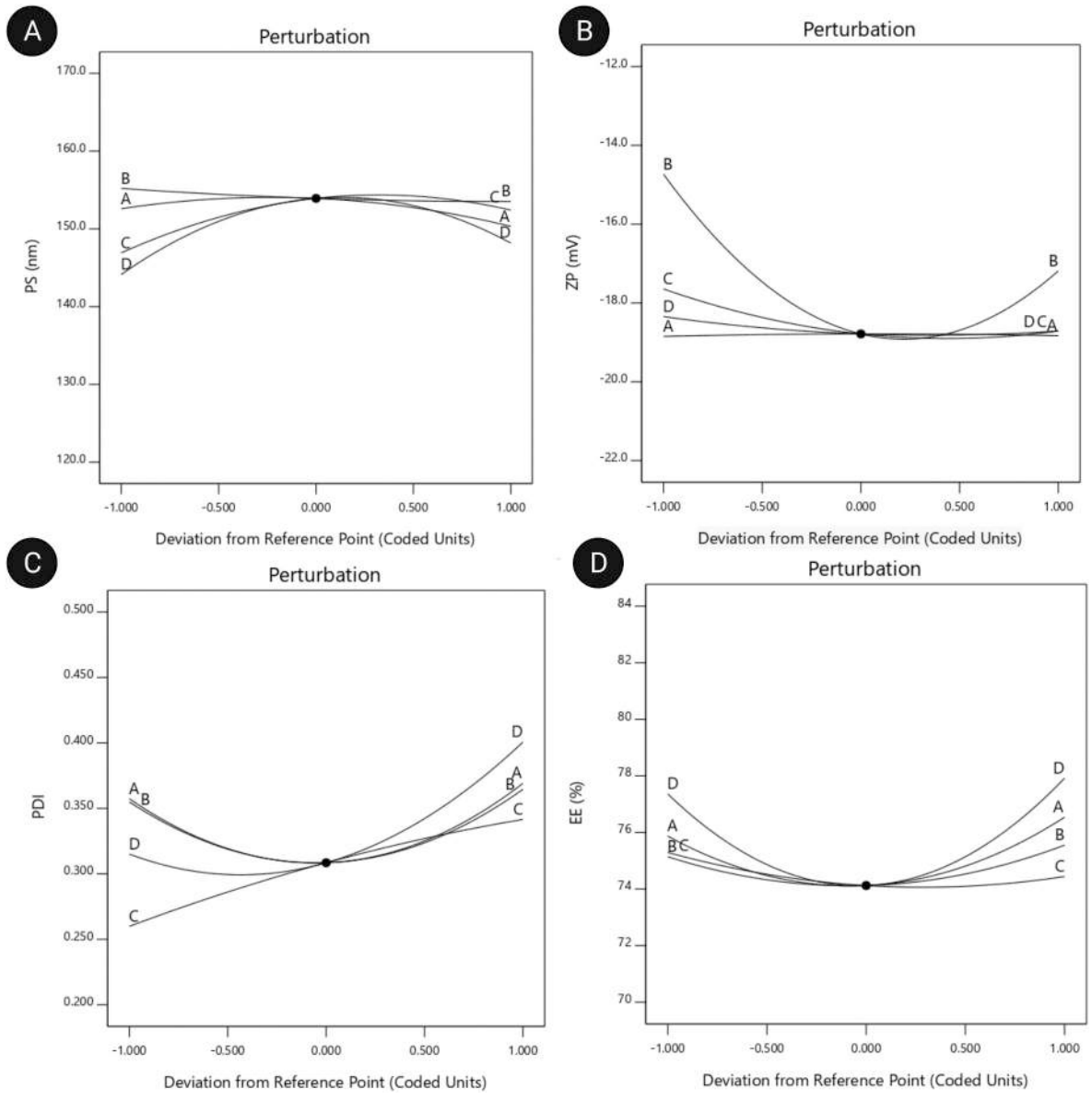


Fig. 4. The perturbation plot for (3.A.) Particle size, (3.B.) Zeta potential, (3.C.) PDI and (3.D.) % Entrapment Efficiency.

Zeta potential (ZP).

The perturbation plot (Fig. 4.B.) revealed that ZP was highly influenced by factor B. As per 2D contour plots (Supplementary Fig. S2·B) and 3D response surface plots (Fig. 5.B), ZP was more affected by A and B factors and less affected by C and D factors. The highest ZP was observed in F24 (−20.5 mV) and lowest ZP in F27 (−13.8 nm). The measured ZP reflected the stability of developed formulations (F1 to F29), in which the physical appearance of NLCs was clear with no signs of instability, such as creaming or cracking. Generally, a ZP greater than ±30 mV is considered adequate to ensure the physical stability of NLCs [72]. However, a charge of ±15 to 20 mV is sufficient to stabilize the formulation by both stearic and electrostatic stabilizers [73].

3.3.2. *Polydispersity index (PDI)*

In perturbation plot (Fig. 4.C) it was observed that PDI was highly influenced by factor D. The graphs of 2D contour plot (Supplementary Fig. S2.C) and 3D response surface plots (Fig. 5.C) indicated that PDI has shown positive effect in response to factors A, B, D and negative effect in response to factor C. The maximum PDI was observed in F2 (0.50) and minimum PDI was observed in F18 (0.21). Salvia-Trujillo and coworkers reported that variation in PDI was due to ultrasound waves used during selected method, which provided multimodal distribution at any amplitude or, power [74]. Also, ultrasounds disrupted larger droplets into smaller ones which led to stabilization of NLCs. This was confirmed by clear appearance of NLCs and absence of phase separation in developed formulations (F1 to F29).

% Entrapment efficiency (% EE).

It was observed that % EE was highly influenced by factor D as per perturbation plot shown in Fig. 4.D. The 2D contour plots (Supplementary Fig. S2D) and 3D response surface plots (Fig. 5.D) shown that response Y2 (% EE) was more influenced by dependent variables A and B as compared to C and D. Higher % EE (Y2) was observed due to low surface tension (because of surfactant and co-surfactant) between droplets that prevented their coalescence, which was further confirmed by the absence of phase separation. This led to enhancement in solubility of 5-FU due to presence of GMS containing long chain solid lipid and its retention in NLCs due to presence of Labrafil M 2125 CS, which avoided leakage of 5-FU [75]. The maximum % EE was observed in F18 (86.0 %) and minimum % EE was observed in F13 (70.2 %).

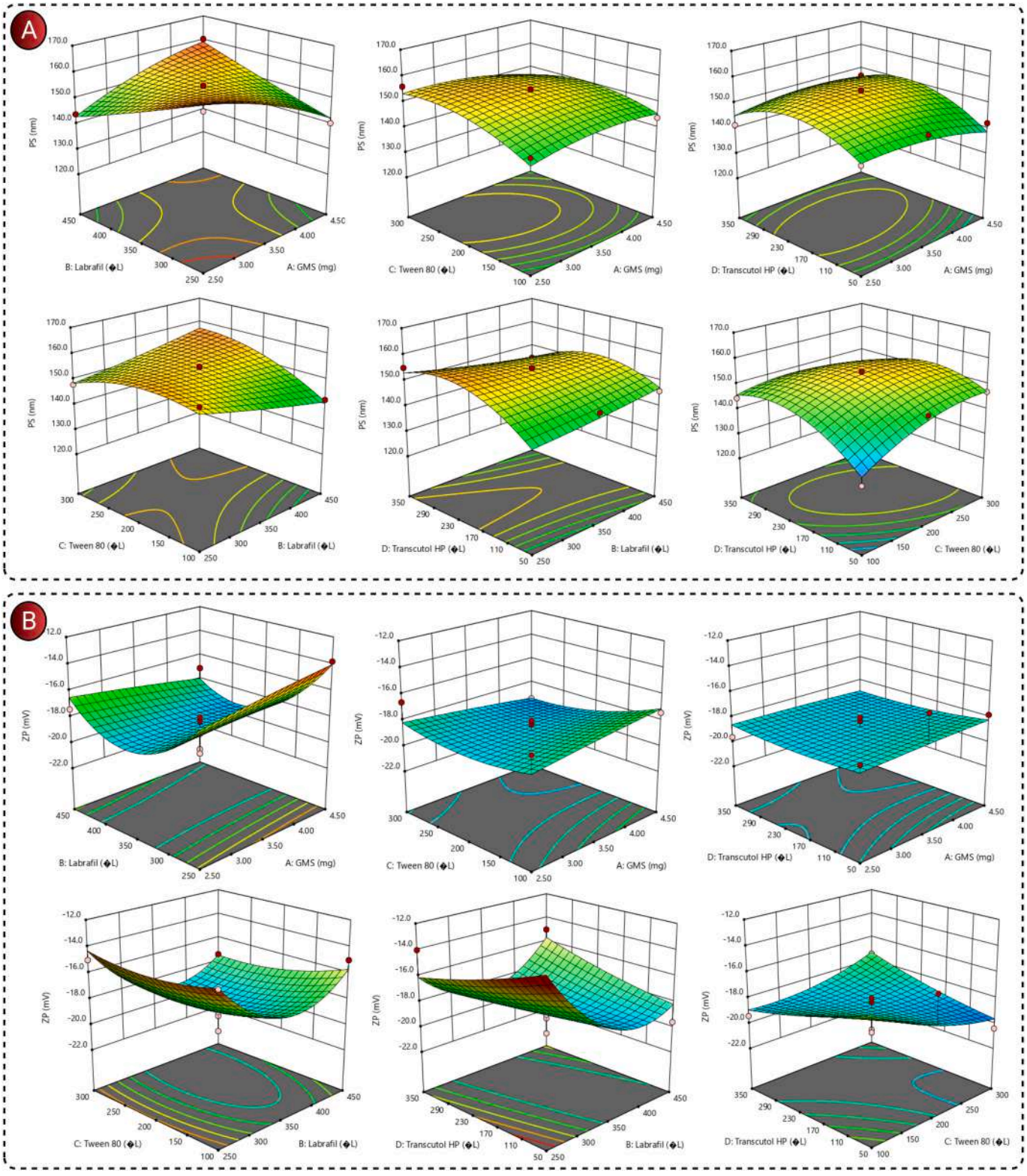


Fig. 5. The 3D response surface plots of (A) Particle size, (B) Zeta potential, (C) PDI and (D) %Entrapment Efficiency.

3.4. Optimization and characterization of 5-FU-NLCs

The formulation variables were optimized by graphical optimization. According to the BBD, a total 29 NLCs prototypes (F1to F29) have been developed and characterized for PS (Y1) and % EE (Y2) as response parameters. The optimized batch was obtained with a composition of GMS, Labrafil M2125 CS, Tween 80, and Transcutol HP as 3.5 mg, 350 µL, 100 µL, and 50 µL, respectively. The overlay plot as per experimental design has been shown in Supplementary Fig.S3. With the use of these predicted values the goal prediction for minimum PS was 129.4 nm, ZP of −17.0 mV, PDI of 0.21 and maximum % EE of 81.0 %. The formulation was labelled as F30. The obtained responses of PS, ZP, PDI and %

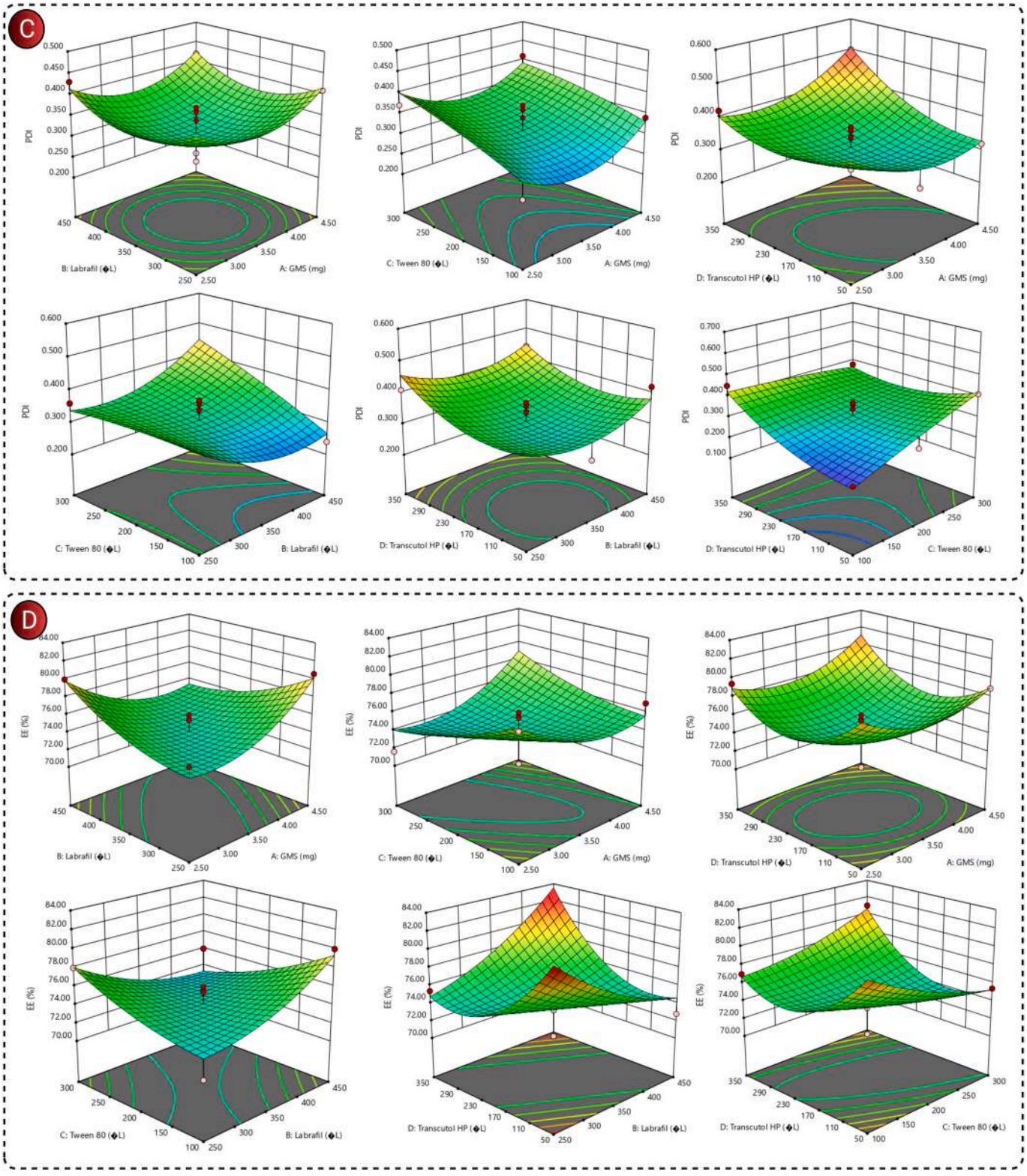


Fig. 5. (*continued*).

EE for optimized batch (F30) were found to be nano size range (131.1 ± 2.3 nm), negative ZP (−16.0 ± 0.5 mV), narrow size distribution (0.26 ± 1.52 PDI) (Supplementary Fig. S4) and high % EE (81.40 ± 0.20 %), respectively. These values were found in concordance with the predicted values. Hence, formulation F30 was used for further surface modification with chitosan (CS) and characterization.

3.5. Characterization of CS-5-FU-NLCs

The surface modification of F30 batch was done with different concentration of CS (0.1 to 0.9 % *w/v*) in 0.1 % *v/v* acetic acid to form F31 to F35 batches of CS-5-FU-NLCs (Table 2). All batches were subjected for PS, ZP, PDI, and % EE and observed to be in the range of 163.2 ± 2.3 nm (F33) to 168.2 ± 1.5 nm (F32) for PS, 19.3 ± 1.4 (F31) to 21.4 ± 0.5

Table 2
Observed responses for CS-5-FU-NLCs.

Formulation code	Chitosan concentration (% *w/v*)	Response 1: Particle size (nm)	Response 2: Zeta potential (mV)	Response 3: PDI	Chitosan concentration (% w/v)
F31	0.1	167.4 ± 1.3	19.3 ± 1.4	0.43 ± 0.32	73.3 ± 2.0
F32	0.3	168.2 ± 1.5	20.1 ± 1.2	0.32 ± 1.32	80.2 ± 2.3
F33	0.5	163.2 ± 2.3	21.4 ± 0.5	0.28 ± 0.22	85.2 ± 0.2
F34	0.7	165.7 ± 1.3	20.7 ± 2.5	0.31 ± 0.12	75.5 ± 1.3
F35	0.9	166.2 ± 1.5	21.5 ± 1.8	0.43 ± 0.23	80.5 ± 2.5

(F33) for ZP, 0.28 ± 0.08 (F33) to 0.43 ± 0.32 (F31) for PDI, and 73.3 ± 2.0 % (F31) to 85.2 ± 0.2 % (F33) for % EE. Based on minimum PS and maximum % EE, F33 batch (CS-5-FU-NLCs) was selected in which surface modification of F30 batch was done with 0.5 % w/v CS. The F33 batch exhibited nano size range (163.2 ± 2.3 nm), narrow size distribution (0.28 ± 0.08 PDI), positive ZP (21.4 ± 0.5 mV) (Supplementary Fig. S5) and high % EE (85.2 ± 0.2 %). Hence, F33 was selected as final batch for further evaluation such as DSC, SEM, TEM, *in vitro, ex vivo,* and *in vivo* studies.

Moreover, it was observed that PS was increased in F33 (CS-5-FU-NLCs, 163.2 ± 1.5 nm) as compared to F30 (5-FU-NLCs, 131.1 ± 2.3 nm) formulation owing to surface modification of uncoated 5-FU-NLCs with CS. However, the lower values of PS and PDI (<0.5) of both F30 and F33 are indicative of uniform PS [76]. CS caused formation of thick multi-layer on NLCs surface leading to enhancement in PS after surface modification of F30 with CS [77]. These results were further confirmed by DSC and SEM studies.

ZP value also changed from negative (−16.0 ± 0.5 mV, F30 batch) to positive (F31 to F35 batches) due to surface modification of F30 with CS to form F31 to F35 batches. This also indicated good coating of positively charged CS on the surface of 5-FU-NLCs. CS is a natural polysaccharide with positive charge, having the ability to adhere to negatively charged mucosal eye surface due to electrostatic interactions, which lead to prolonged residence time at drug absorption sites [25]. CS has been most widely used for topical delivery of drug to the posterior eye's segment and acted as absorbefacient for ophthalmic dosage forms by adhering with negatively charged corneal surface [45].

Maximum % EE was observed after surface modification of F30 with CS in F33 batch due to increase in PS of 5-FU-NLCs (F30), which allowed higher entrapment of 5-FU. Similar findings were reported by Gilani et al. for CS coated Luteolin NLCs which shows higher % EE as compared to uncoated Luteolin NLCs due to coating with CS [78].

3.6. FTIR spectra analysis

FTIR spectrum of 5-FU showed sharp characteristic peaks at 3064.99 cm^{-1} (N—H stretch), 1645.33 cm^{-1} (C—N stretch), and 462.93 cm^{-1} (C—H stretch). FTIR spectrum of 5-FU-NLCs also showed characteristic peaks at 3331.18 cm^{-1} (N—H stretch), 1637.62 cm^{-1} (C—N stretch), and 455.22 cm^{-1} (C—H stretch) pertaining to the peaks of 5-FU. There was no significant shift in the vibrational frequencies of 5-FU upon loading into NLCs. This indicated that the drug was compatible with the excipients used to prepare NLCs. However, the obtained peaks were smooth with less intensity, indicating the entrapment of 5-FU in the globules made up of lipid and surfactants [47]. FTIR spectrum of CS showed sharp characteristic peaks at 3429 cm^{-1} (CH2 stretch), 1652.67 cm^{-1} (C═O stretch), 1380.98 cm^{-1} (C-O-C stretch), and 605.03 cm^{-1} (N—H bend). Similar to the results of F-FU-NLCs, FTIR spectrum of CS-5-FU-NLCs showed characteristic peaks at 3292.6 cm^{-1} (N—H stretch), 1635.69 cm^{-1} (C—N stretch), 1247.99 cm^{-1} (C-O-C stretch), and 437.86 cm^{-1} (C stretch) pertaining to 5-FU. However, the peaks were smooth with less intensity even as that of FTIR spectrum of 5-FU-NLCs deciphering the coating of CS over the NLCs. Thus leading to surface modification [48]. The results are shown in Fig. 6A and supplementary Table S5.

3.7. DSC

DSC analysis of 5-FU, CS, optimized batch of 5-FU-NLCs (F30) and CS-5-FU-NLCs (F33) was performed to observe the physicochemical changes occurred during formulation development. In DSC thermogram of 5-FU endothermic peak was observed in 284.52 °C, but in case of F30 and F33, 5-FU peak was disappeared, which indicated 5-FU got solubilized into solid lipid (GMS) and encapsulated inside the lipid matrix. In both batches *i.e.* F30 and F33, exothermic peak was observed at 69.01 °C due to evaporation of water from prepared NLCs. In DSC curve of CS, exothermic peak was observed at 300 °C due to decomposition of amine group and endothermic peak was observed at 100 °C due to loss of water molecule. DSC thermograms are shown in Fig. 6B.

3.8. SEM

The SEM images of optimized batch of 5-FU-NLCs (F30) and CS-5-FU-NLCs (F33) were shown in Fig. 7. The image of F30 (Fig. 7A) appeared to be spherical and showed smooth surface with PS <100 nm. Whereas, in case of F33 (Fig. 7B) irregular surface was observed due to surface modification of chitosan (CS) on F30. The structure of F33 was appeared to be elongated with coarse surface, asymmetrical and smooth boundaries as compared to F30. Thus, the outcomes of SEM for developed F30 and F33 formulation justified the surface modification of F33 with CS.

3.9. TEM

The TEM analysis of optimized batch of 5-FU-NLCs (F30) and CS-5-FU-NLCs (F33) (Fig. 7) was performed to observe the surface characteristic and morphological observation. In Fig. 7C, TEM image of F30 revealed spherical and unagglomerated particles in nanometer range with average diameter of 100 nm ± 1.02 nm as per PS scale bar 500 nm. In Fig. 7D, F33 shown irregular surface particles in nanometer range with average diameter of 180 nm ± 1.23 nm size (<200 nm) due to surface modification by CS in F33 formulation.

3.10. Robustness to pH change

The results of PS and zeta potential analysis indicated no significant change among their results with respect to their results in water as the *p* value between their results was found <0.05. This indicated that the NLCs remain un-agglomerated/unaffected with change in pH. The results are shown in Fig. 8. A slight decrease in particle size was observed in case of CS-5-FU-NLCs diluted in pH 7.4 phosphate buffer as compared to the CS-5-FU-NLCs diluted in water. Whereas, the zeta potential was found slight higher (22.89 ± 1.19) in acetate buffer (pH 4.5) as compared to any of the medium. Overall, no significant change was observed in case of zeta potential and particle size analysis of NLCs upon changing the medium having different pH.

As the CS-5-FU-NLCs have been administered through ocular route, the particle size plays an important role in their transport from the site of application to the retina by having the deeper penetration property due to size <200 nm. The smaller particle size will allow the drug to pass though the ocular barriers and help in deeper penetration of 5-FU. The

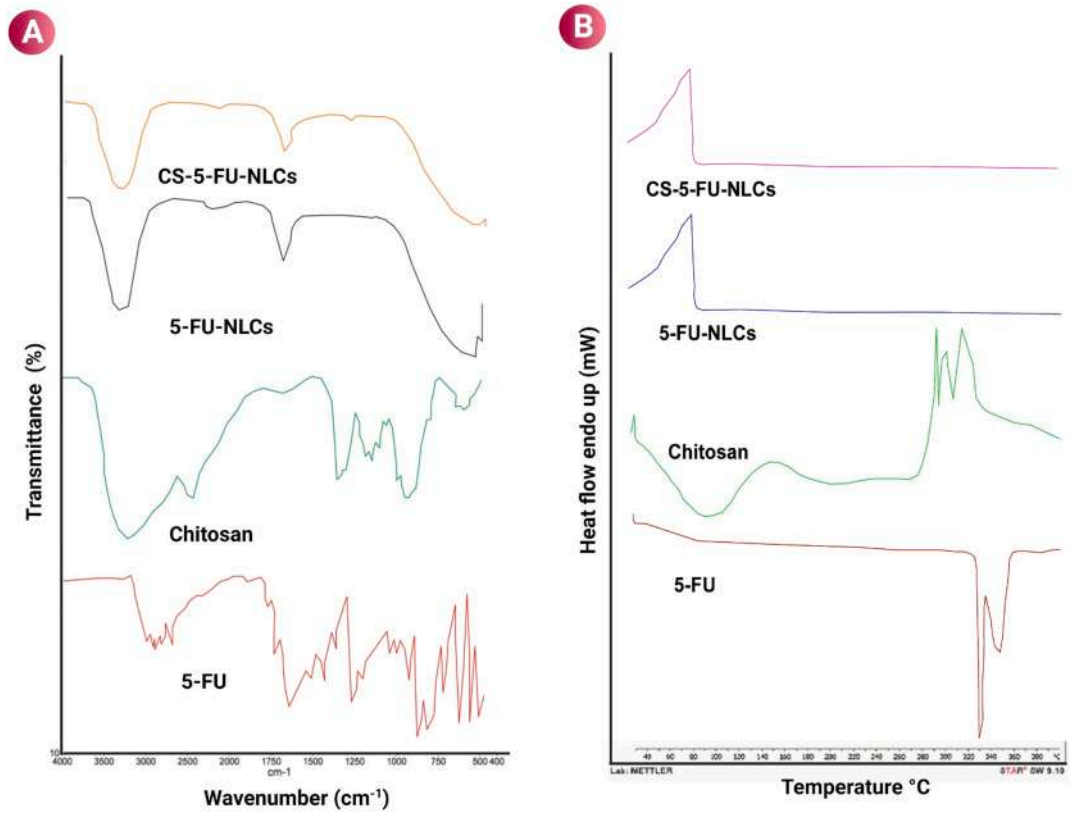


Fig. 6. (A) FTIR spectra and (B) DSC thermograms of 5-FU, Chitosan, 5-FU-NLCs, CS-5-FU-NLCs.

size of particle <200 nm is generally recommended for ocular administration [79].

3.11. *In vitro* drug release study and kinetic modelling

Percentage cumulative drug release (±SD) was calculated ($n = 3$) for batch-I (5-FU solution), batch-II (F30, 5-FU-NLCs), and batch-III (F33, CS-5-FU-NLCs) formulations. The graph was plotted between % cumulative drug release *versus* time, as shown in Fig. 9A. *In vitro* drug release of batch-I exhibited rapid release of 5-FU with almost 98.5 ± 0.3 % within first 2 h whereas drug release profiles from batch-II and III were found to be sustained and 97.3 ± 0.2 % and 81.0 ± 0.4 %, respectively in 48 h. The results of batch II and III shown sustained release profile of 5-FU over the time period of 48 h as compared to batch-I. However, in case of batch-III comparatively higher delay in drug release profile of 5-FU was observed owing to the swelling property and mucoadhesive nature of CS, which can form swellable matrix layer around 5-FU-NLCs leading to its controlled drug release *viz.* diffusion, swelling, and erosion of CS matrix [80]. The drug release profiles of batch-I, II and III were compared using one way ANOVA. The p value suggesting significant differences in the release profile of 5-FU in batch-III as compared to batch I ($p < 0.01$) and batch-II ($p < 0.05$) [81]. Similar findings are reported in another study which mentioned that sustained release effect of surface modified NLCs with CS was observed due to encapsulation of drug in the inner core of the lipid matrix which, then released slowly by diffusion [78]. It is important to note that due to structural similarity of CS to the physiological glycosaminoglycans it gets easily degraded *in vivo* by the hydrolytic action of lysozyme. This enzyme is non-specific in nature and largely present in the mucus. CS is highly susceptible to enzymatic depolymerization as compared to other polysaccharides leading to degradation products that include D-glucosamine, *N*-acetyl-glucosamine and *N*-acetyl-glucose. All these products are nontoxic to the human body. In addition, CS's degradation intermediates also do not give rise to problems of accumulation in the body and do not have immunogenic power. This allows the safe administration and degradation of topically applied CS-based mucosal delivery systems [82].

The results of kinetic modelling for batch-II and III are shown in Table 3, which indicated controlled release of 5-FU from batch-III *vis-a-vis* batch-II. However, batch-II formulation showed highest R^2 value *i.e.* 0.978 with Korsmeyer-Peppas model which indicated that 5-FU was diffused from lipid matrix in control release mechanism [49]. Whereas, in batch-III after surface modification of 5-FU-NLCs with CS, around 1.7-fold augmentation in rate of 5-FU release was observed as compared to batch-II. Also, in batch-III highest R^2 (coefficient of regression) value (0.990) was found with Hixon-crowell model which indicated that release of 5-FU was dependent on change in diameter and surface area of CS-5-FU-NLCs, owing to mucoadhesive property of CS which formed swellable matrix layer around CS-5-FU-NLCs [83]. The value of kinetic release coefficient (n) was found 0.561 for batch-II and 0.631 for batch-III, thereby exhibiting non-Fickian diffusion owing to drug diffusion and lipid matrix erosion. Hence, it can be concluded that in batch-III, 5-FU release was primarily through diffusion mechanism, plausibly owing to hydrophilic nature of 5-FU. This was followed by complete biodegradation of CS after releasing its contents [84].

3.12. *In vitro* ocular irritation studies using HET-CAM model

HET-CAM assay is a good model of choice to study ocular irritation study. The results of *in vitro* ocular irritation study and irritation score (IS) of group-I (positive control, 0.1 N sodium hydroxide), group-II (5-FU solution), group-III (F30, 5-FU-NLCs) and group-IV (F33, CS-5-FU-NLCs) were calculated as per Eq. (2) and shown in Fig. 10. The results ($n = 3$) revealed that developed formulations *i.e.* groups - II, III and IV showed no signs of vascular injury or coagulation and found non-irritant as compared to group-I that showed severe irritation. Further, IS was calculated by observed reactions of lysis, haemorrhage, and coagulation

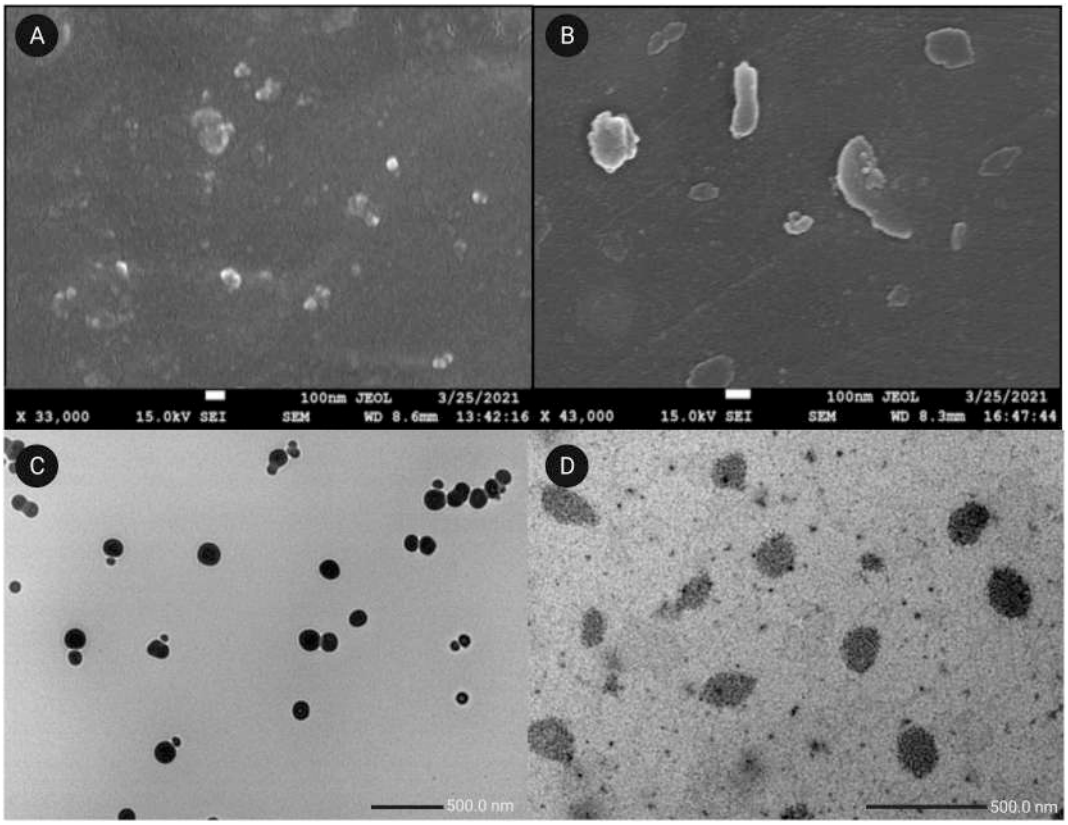


Fig. 7. SEM images (scale bar: 100 nm) of A. 5-FU-NLCs (F30) and B. CS-5-FU-NLC (F33) and TEM images (scale bar: 500 nm) of C. 5-FU-NLCs (F30) and D. CS-5-FU-NLC (F33).

on the CAM over a period of 6 h and net IS being calculated. The value of IS was "20.56" for group-I and for group-II, III and IV it was "0". Hence, non-irritant nature of 5-FU was observed in group-II, III and IV with good ocular tolerance (n = 3) in comparison to group-I [85].

3.13. Ex vivo permeability study using goat cornea

Ex vivo permeability study was performed upto 4 h and then percentage permeation of 5-FU (± SD) was calculated (n = 3) for batch-I (5-FU solution), II (5-FU-NLCs, F30), and III (CS-5-FU-NLCs, F33). After 4 h, from batch-I, II and III, around 56.2 ± 2.5 %, 71.2 ± 1.4 %, and 80.9 ± 2.2 % respectively 5-FU got permeated. The graph was plotted between percent permeation of 5-FU *vs.* time and shown in Fig. 9B. The results of permeation study for batch-I, II, and III were compared by one-way ANOVA. The p values were <0.05 indicating significant differences in 5-FU permeation in case of batch-III as compared to batch-I ($p < 0.01$). Moreover, in batch-III it got further improved nearly by 10 % ($p < 0.05$) as compared to batch-II. Similar findings have been reported in another study, wherein it was mentioned that surface-modified form of drug loaded NLCs with CS got adhered firmly with mucin and showed improved trans corneal permeability [86].

3.14. In vivo studies

3.14.1. Anti-angiogenesis study by chorioallantoic membrane (CAM) assay

In anti-angiogenic study, the blood vessels of eggs were arranged in a tree-like (dendritic) branching pattern with an equal distribution, covering the entire area of the CAM in case of eggs of group-I receiving positive control- sodium pyruvate) (Fig. 11). The vascular architecture of the CAM appeared to be originating from the main "λ" branch of the blood vessel *i.e.,* central vessel of egg which further differentiated into collateral vessels *viz.* primary, secondary, and tertiary branches.

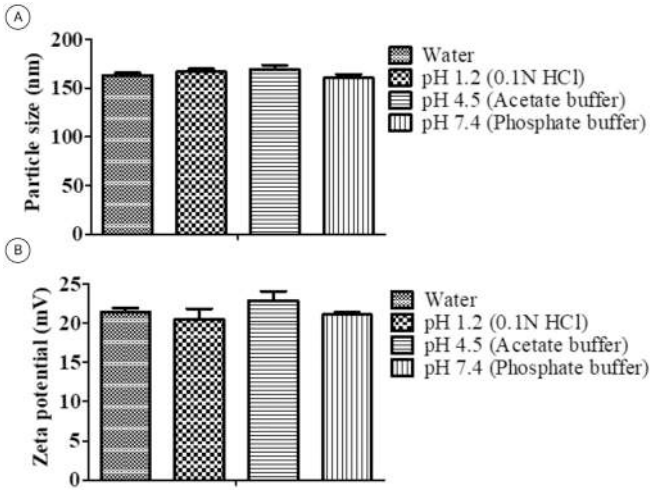


Fig. 8. Results showing effect of change in pH on size and zeta potential of CS-5-FU-NLCs.

Subsequently, increase in growth of alternative collateral vessels was seen for survival of embryos [87]. As the inoculation of respective formulations in group II, III, and IV eggs were carried out in air cavity of chicken eggs, the immediate effects were occurring on the central vessel development. In case of group-II, III and IV eggs, central vessel 's growth was largely compromised due to anti-angiogenesis effect of 5-FU [17]. The number of blood vessels of eggs were counted for group-I (97.0 ± 1.0), group-II (49.0 ± 2.0), group-III (44.0 ± 2.0) and group-IV (33.0 ± 1.0) as mean (± SD). The graph was plotted between mean count of blood vessels of eggs *vs.* groups. All groups were compared using one-

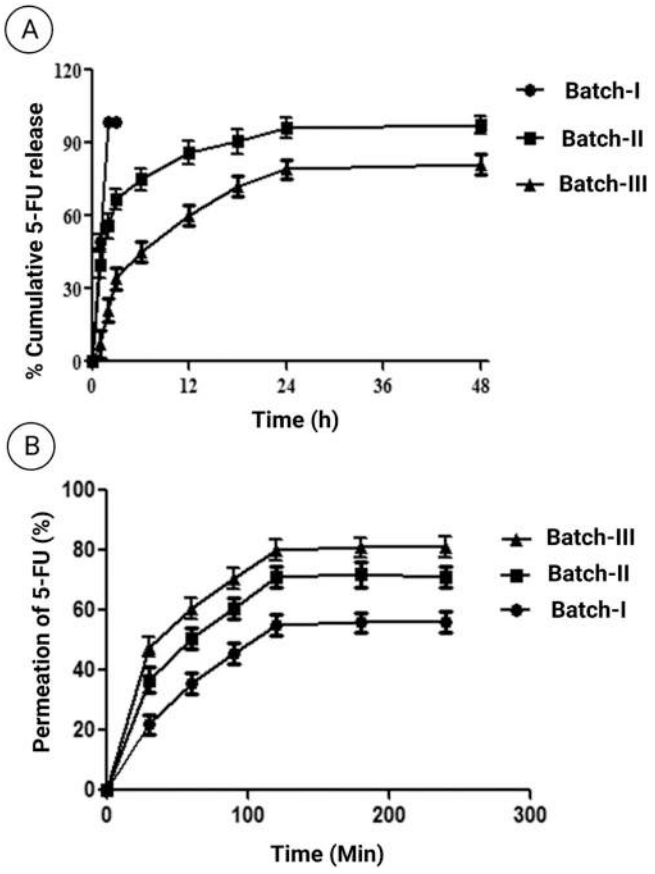


Fig. 9. A. *In vitro* drug release of batch-I, II and III ($n = 3$) mean ± SD; B. Permeation study of Batch-I, II and III (n = 3) mean ± SD.

Table 3
Kinetic modelling drug release data.

Models	Batch-II R^2[a]	Interpretation	Batch-III R^2	Interpretation
Zero order	0.742	–	0.961	–
First order	0.957	–	0.989	–
Korsmeyer-Peppas	0.978	● Sustained release profile of 5-FU from lipid matrix ● Non-Fickian diffusion	0.924	–
Higuchi model	0.947	–	0.961	–
Hixson Crowell	0.903	–	0.990	● Delayed release profile of 5-FU, due to chitosan swelling layer around CS-5-FU-NLCs and mucoadhesion property ● Non-Fickian diffusion

[a] R^2 (Coefficient of regression).

way ANOVA as shown in Fig. 11. The p value suggested significant reduction in mean count of blood vessels for group-II ($p < 0.01$), group-III ($p < 0.01$) and group-IV ($p < 0.001$) as compared to group-I.

3.14.2. *In vivo studies in a diabetic retinopathy (DR) rat model*

Angiogenesis is development of new blood vessels from existing one's which may later on leads to DR in case of eyes. For prevention of DR, anti-angiogenic drug plays an important role in management of DR. Different *in vivo* models have been reported to evaluate the anti-angiogenic effect of drug such as rat aortic ring assay [88], anti-neovascularization model [89], DR rat model [58] *etc.*

Rat aortic ring assay and anti-neovascularization model are most extensively used methods to validate the anti-angiogenic effect [88]. But DR is not induced in these model, instead of that vascular endothelial growth factor (VEGF) was used and anti-VEGF effect of drug was determined [90]. VEGF has a potent role in angiogenesis, which plays direct effects on vascular endothelial cells, including tubulogenesis, endothelial cell proliferation, and vascular permeability [91]. In one of the studies, rat aorta was used to check cell proliferation, migration, and aorta tube formation after treatment with itraconazole (ITR). It has shown the absence of sprouting of vessel growth which proved anti-angiogenic effect of ITR [12].

Another, *in vivo* anti-neovascularization model also reported for non-invasive evaluation of anti-angiogenic effect. In this study, iris angiogenesis in the rat's eye was observed directly in clinical diagnostics, suggesting that animal models of iris angiogenesis could be easily evaluated and quantified *in vivo* by noninvasive methods [89]. In another method, corneal neovascularization was induced by alkali injury on rat eyes and anti-angiogenic effect was determined [92]. However, in these methods, no direct induction of DR reported.

Diabetes induced DR rat model is also reported in which single intraperitoneal injection of streptozotocin (STZ, 35 mg/kg) in PBS is injected into rats and progression of DR is observed after 3 month of DM development [93]. After 72 h of the STZ injection, blood glucose levels (BGL) were monitored to ensure DM development in rats, which is further continued for next 10 weeks for successful development of DR and its associated complications including astrocyte defects, increased neuronal cell death, microglial cell activation, and microvascular leakage in rat models of T1DM [58]. At the end of the 10th week, approximately 80 μL of the respective formulations were administered as eye drops in rat's eyes once a day for 20 days. After 20 days, electroretinography (ERG) was performed to ensure DR in live rats. After this, the group of rats were sacrificed on day 21st of treatment and isolated eye balls were analyzed for histopathological examination [56].

The STZ induced model is better as compared to others (rat aortic ring assay and anti-neovascularization model) as it involves induction of diabetes in rats which later on leads to DR after 3 months. Hence, a real picture of DM based DR appears and anti-angiogenic effect of 5-FU was observed in rat's eyes primarily by fungus imaging and later on confirmed by histopathological examination.

3.14.2.1. Fundus imaging. Images of rat's eyes were captured for group I, II, III, and IV on the 91st day (after 3 months of DM induction) and are shown in Fig. 12. These images were taken before giving any treatment to diabetes induced DR rats (to ensure development of retinopathy in rats) and after 20 days' treatment given to group-II, III and IV rats. In group I rats-The retinal blood vessels were seen with an equal distribution in eye, covering the entire retinal area in group-I rats whereas after topical application of 5-FU solution, F30 and F33 formulations in the form of eye drops in right eye (RE) of group II, III and IV rats respectively for 20 days, rat retinal vessel's thickness were found to be

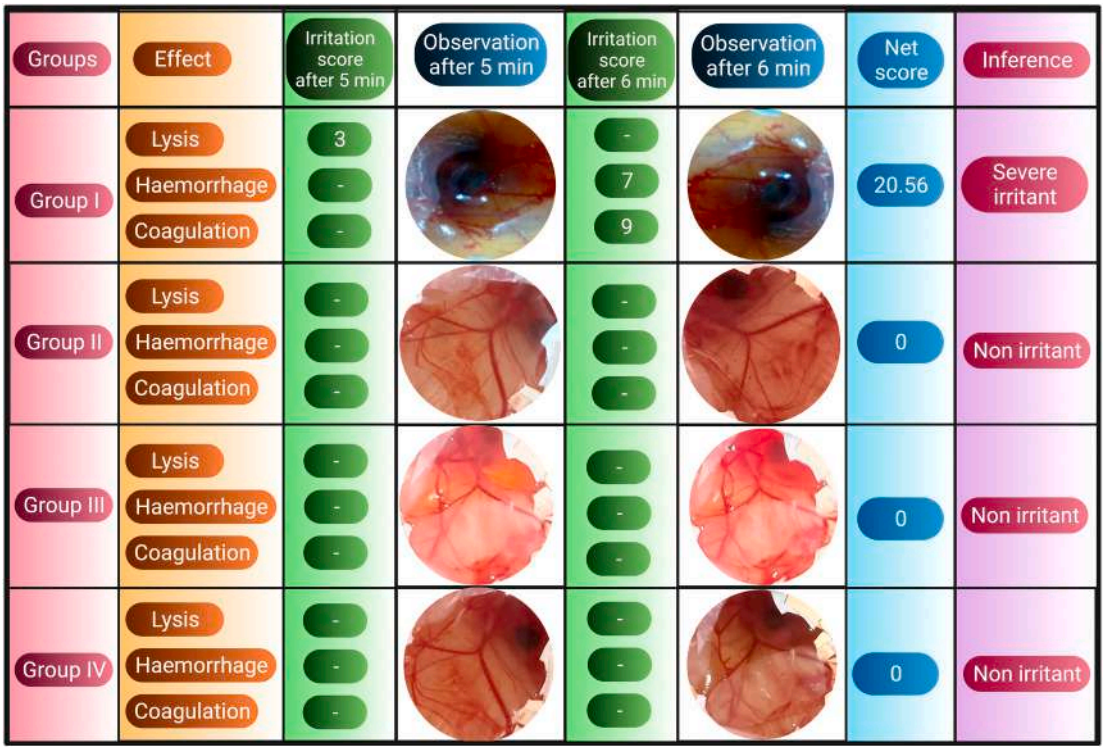


Fig. 10. Ocular irritation scores and CAM images of group I, II, III and IV.

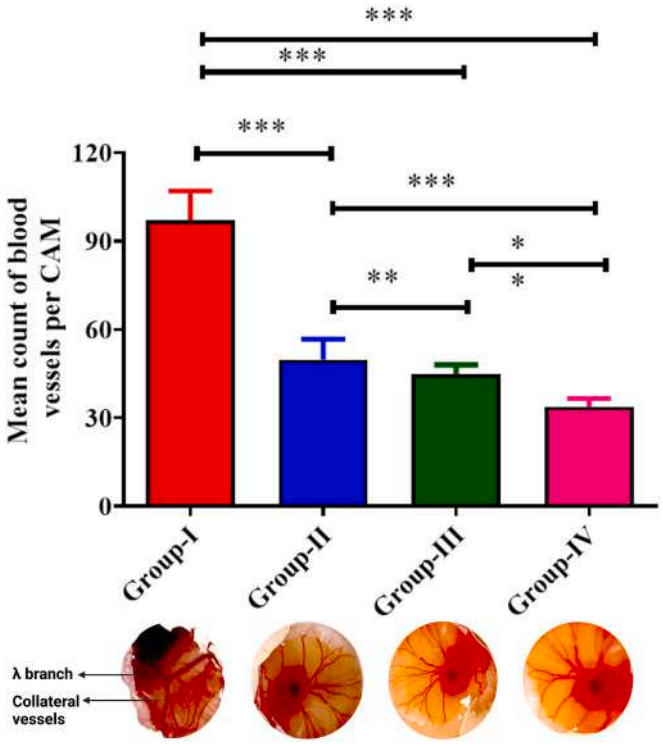


Fig. 11. Results of anti-angiogenesis study by CAM assay: Mean ± SD blood vessels of group-I, II, III and IV (*** $p < 0.001$, ** $p < 0.01$).

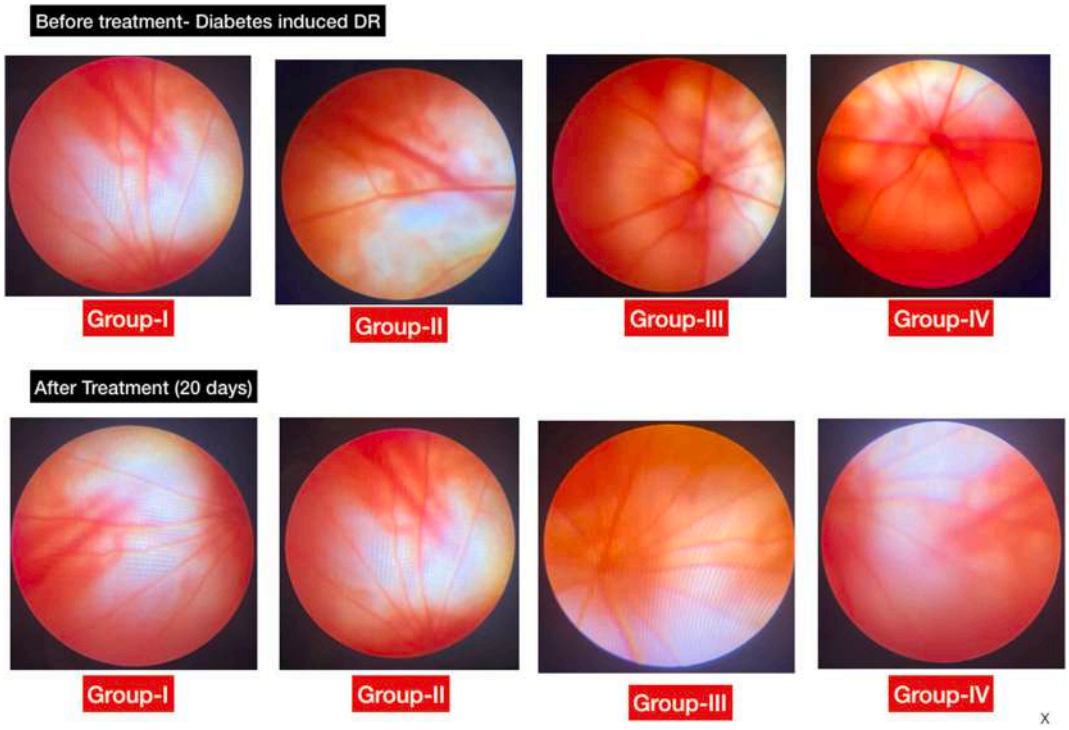


Fig. 12. Electroretinographs of groups-I, II, III and IV.

thick. In addition, there was increase in number of retinal vessels *i.e.,* angiogenesis due to DM induced DR. The immediate effects were seen *via* decrease in thickness and number of retinal blood vessels *i.e.,* anti-angiogenesis in group-II, III and IV rat eyes due to angiogenesis inhibitory activity of 5-FU.

3.14.2.2. Histopathological examination. The observations of ERG were further confirmed by histopathological images captured for retinas of group I, II, III, and IV rats are shown in Fig. 13A. The retinal vessels thickness of each group was measured. The normal retinal vessel thickness was found to be 150.0 ± 2.3 mm, whereas in case of group-I, angiogenesis was observed and it was increased upto two times *i.e.*, 300.0 ± 1.3 mm. However, decrease in thickness of retinal vessels were observed after topical application of 5-FU solution, F30 and F33 formulations in right eye of group II, III and IV rats respectively for 20 days. In group II, thickness of rat retinal vessels was found to be 220.0 ± 1.5 mm, in group III it was 180.0 ± 1.1 mm and in group IV it was 160.0 ± 2.3 mm, which indicated anti-angiogenic effect of 5-FU as shown in Fig. 13B. All results were compared using one-way ANOVA. The p value suggested significant reduction in retinal vessels thickness for group-II ($p < 0.01$), group-III ($p < 0.01$) and group-IV ($p < 0.001$) as compared to group-I.

All the obtained results indicated that F33 formulation offered successful delivery of 5-FU to the retina by improving its permeability at desired site.

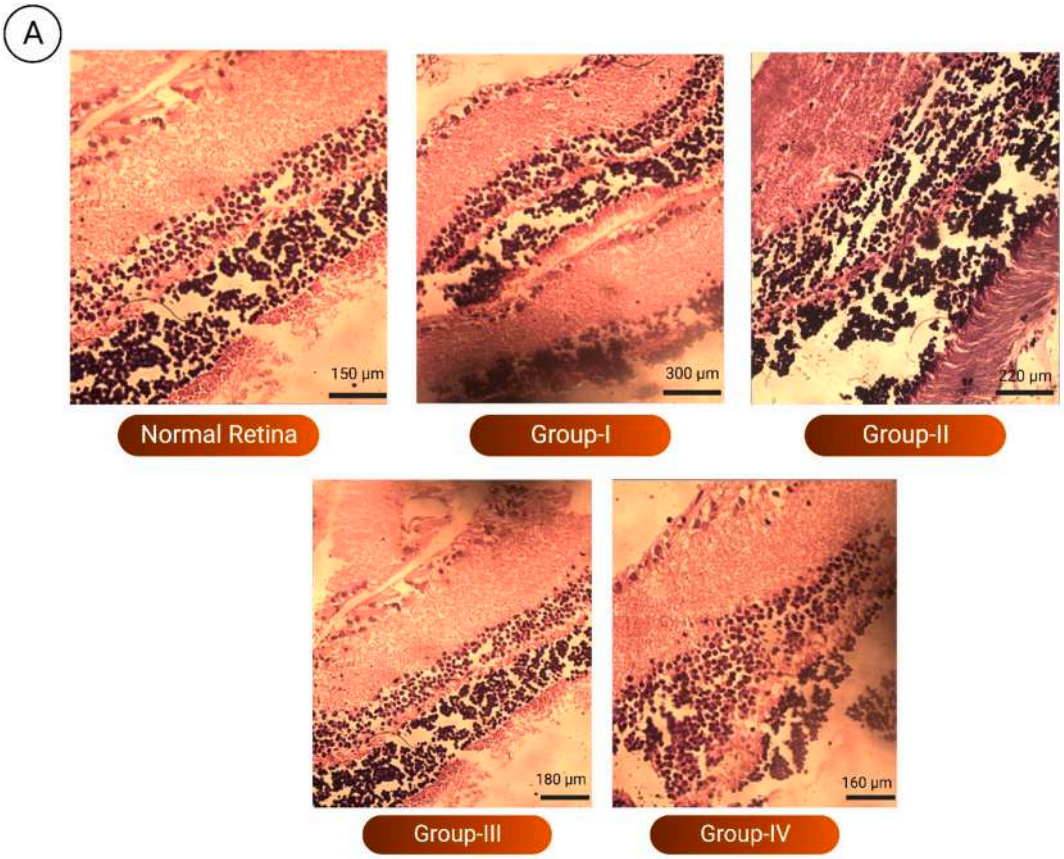
3.15. Accelerated stability studies

This study was performed using accelerated storage conditions such as temperature and relative humidity as part of the stability testing to ensure the stability of final formulation F33 [62]. The F33 formulation was evaluated ($n = 3$) for PS, ZP, PDI, and % EE at various time intervals (0, 1st, 3rd and 6th months). The PS (164.7 ± 3.2 nm), PDI (0.22 ± 0.11), and % EE (81.3 ± 2.5 %) of CS-5-FU-NLCs (F33) were found stable, during the storage period of 6 months as evident from Table 4. There was a slight decrease in ZP (20.1 mV ± 1.9 mV) upon storage. However, this decrease was non-significant as compared to fresh formulation.

4. Conclusion

In the present study the CS-5-FU-NLCs were formulated with the help of BBD. The size, polydispersity index, zeta potential and entrapment efficiency of CS-5-FU-NLCs were 163.2 ± 2.3 nm, 0.28 ± 1.52, 21.4 ± 0.5 mV and 85.0 ± 0.2 %, respectively. These size and zeta potential remain statistically unchanged upon change in pH of medium used to prepare NLCs, indicated towards their robustness. A release of 5-FU about 98.5 ± 0.3 % from batch I within first 2 h and 97.3 ± 0.2 % and 81.0 ± 0.4 % from NLCs and CS modified NLCs, respectively in 48 h indicated controlled release of drug. The CAM assay showed non-irritant nature of developed 5-FU NLCs. Furthermore, the *in vivo* antiangiogenic study performed on streptozotocin induced DR rats showed excellent anti-angiogenesis potential of CS-5-FU-NLCs.

Overall, the present research showed the anticancer drug 5-FU can be utilized as an anti-angiogenic agent for the treatment of DR. Additionally, the study entailed that NLCs can be an ideal carrier for treating DR through ocular route. Moreover, the surface modification of 5-FU-NLCs using CS can offer controlled release of 5-FU, that would reduce its frequency of administration. The current treatment strategy has offered a new clinical dimension to 5-FU by repurposing it for DR as well as offered a potent non-invasive as well as economical treatment The obtained results from *in vivo* studies on STZ induced DR rats showed



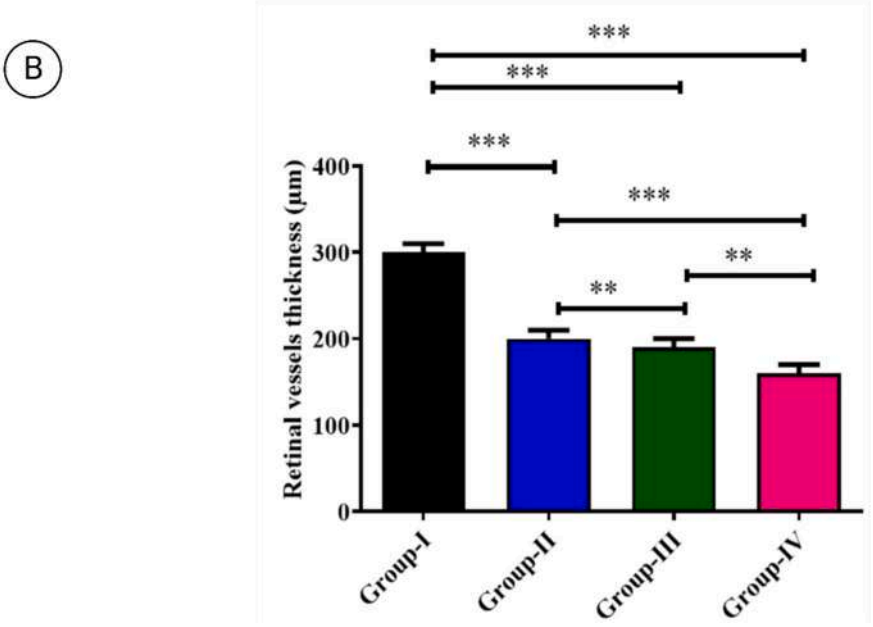


Fig. 13. (A) Histopathological examination of group-I, II, III and IV, (B) Retinal vessel thickness of group-I, II, III and IV (***$p < 0.001$, ** $p < 0.01$).

Table 4
Stability studies of CS-5-FU-NLCs.

Parameters	Storage time in months			
	0	1st	3rd	6th
Physical appearance	Clear and stable	Clear and Stable	Clear and Stable	Clear and Stable
PS (nm)	163.2 ± 2.8	164.4 ± 3.2	164.3 ± 2.1	164.7 ± 3.2
ZP (mV)	21.4 ± 1.6	20.2 ± 1.4	20.1 ± 1.5	20.1 ± 1.9
PDI	0.23 ± 0.51	0.22 ± 0.31	0.22 ± 0.21	0.22 ± 0.11
% EE	81.5 ± 1.5	81.4 ± 0.5	81.4 ± 2.4	81.3 ± 2.5

excellent preclinical efficacy of CS-5-FU-NLCs. However, the results require good clinical correlation because of some limitations of the study. For example, the blinking frequency of rodents is 0.0013 Hz, while it is 4–8 Hz in humans. This means that removal of the formulation from the cornea is expected to be much faster than in the rodent model. Such bottlenecks require studies on humans for exact clinical outcomes. Nevertheless, this research has provided a good dimension and the first important approach to the formulation scientists working in ocular delivery, particular DR.

CRediT authorship contribution statement

Deep Shikha Sharma: Methodology, Data curation, Writing – original draft. **Sheetu Wadhwa:** Supervision, Writing – review & editing. **Monica Gulati:** Writing – review & editing. **Bimlesh Kumar:** Methodology. **Nitin Chitranshi:** Methodology. **Vivek Kumar Gupta:** Methodology. **Mohammed Alrouji:** Methodology. **Sharif Alhajlah:** Methodology. **Othman AlOmeir:** Methodology. **Sukriti Vishwas:** Methodology. **Rubiya Khursheed:** Methodology. **Sumant Saini:** Methodology. **Ankit Kumar:** Methodology. **Shaik Rahana Parveen:** Methodology. **Gaurav Gupta:** Methodology. **Flavia Zacconi:** Methodology. **Dinesh Kumar Chellappan:** Methodology. **Andrew Morris:** Methodology. **Raimar Loebenberg:** Methodology. **Kamal Dua:** Methodology. **Sachin Kumar Singh:** Conceptualization, Validation, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijbiomac.2022.10.168.

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Recent advances in intraocular and novel drug delivery systems for the treatment of diabetic retinopathy

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Abstract

Introduction: Diabetic retinopathy (DR) is associated with damage to the retinal blood vessels that lead eventually to vision loss. The existing treatments of DR are invasive, expensive, and cumbersome. To overcome challenges associated with existing therapies, various intraocular sustained release and novel drug delivery systems (NDDS) have been explored.

Areas covered: The review discusses recently developed intraocular devices for sustained release of drugs as well as novel non-invasive drug delivery systems that have met varying degree of success in local delivery of drugs to retinal circulation.

Expert opinion: The intraocular devices have got very good success in providing sustained release of drugs in patients. Development of NDDS and their application through ocular route has certainly provided an edge to treat DR over existing therapies such as anti-VEGF administration but their success rate is quite low. Moreover, most of them have proved to be effective only in animal models. In addition, extent of targeting the drug to retina still remains variable and unpredictable. Toxicity aspect of the NDDS have generally been neglected. In order to have successful commercialization of nanotechnology-based innovations well designed clinical research studies need to be conducted to evaluate their clinical superiority over that of the existing formulations.

Keywords: Diabetic Retinopathy; Intraocular devices; Novel drug delivery system, Vascular Endothelial Growth Factor

Article highlights

- DR is one of the complications among people suffering from DM
- DR occurs due to high blood glucose level, activation of chronic, low-grade inflammatory signals, and metabolic dysfunction
- Advent of intravitreal anti-VEGF agents has provided better management of DR but gained limited clinical success
- The available intravitreal injections are costly, painful and require skilled person for its administration
- NDDS have been successful in pre-clinical studies due to their non-invasiveness, cost effectiveness and ability to target the drug to retina through ocular route
- Clinical translation of these NDDS of drugs used for treatment of Dr is yet to materialize

1. Introduction

Diabetes Mellitus (DM) is a metabolic disorder that occurs due to elevated blood glucose level. DM is broadly categorized into two types- Type-1 (T1DM) and Type-2 (T2DM). Over 422 million people worldwide have diabetes and almost 1.6 million deaths are directly related to diabetes each year. The global prevalence of diabetes is more than 8.5% in the population over 18 years. The number of diabetic people is expected to increase to 700 million by 2045 [1]. Diabetic retinopathy (DR) is a common co-morbidity among patients having prolonged history of DM. It damages retinal blood vessels and retinal nerves and is considered as a leading cause of vision loss or complete blindness in the diabetic population worldwide. DR is classified into two types: proliferative diabetic retinopathy (PDR) and non-proliferative diabetic retinopathy (NPDR). In PDR, neovascularization (abnormal growth of the blood vessels) of

ocular blood vessels takes place, which leads to sudden vision loss. NPDR is further divided into three categories i.e. mild, moderate and severe NPDR. In mild NPDR, microaneurysms (balloon-like swelling) occur that damage the small retinal blood vessels. In moderate NPDR, blood vessels of the retina get blocked causing deficiency in the supply of oxygen and nutrients required for normal retinal functioning. In severe NPDR, many blood vessels of the retina get blocked and severe oxygen and nutrient depletion happens at the level of retina. Prevalence of anatomical and physiological retinal eye barriers poses a challenge to treatment of DR. Success of any treatment of DR depends on the targeting of the drug to the posterior segment of the eye i.e. by crossing ocular barriers and reaching the retina for the management of DR [2].

In particular, ocular diseases have been treated by two primary modalities i.e. topical drops and intravitreal injections. Since decades topical drops have been a mainstay, however, the compliance has become a major challenge to efficacious therapy about 33% of patients undergoing therapy have been reported to discontinue their therapeutic schedule after one year [3,4]. Introduction of intraocular injections has provided the first effective back-of-the-eye therapy. The approval of ranibizumab and aflibercept for the treatment of wet age-related macular degeneration (wAMD) have provided a breakthrough in intraocular drug delivery. However, in recent studies it has been reported that use and injection frequency did not replicate the trial outcomes in most cases due to insufficient administration frequency [3,5]. The discovery of implantable intraocular devices such as Vitrasert®, Retisert®, Ozurdex® and Iluvein® have been able to provide long term residence within the eye [6,7]. Till date these intraocular devices have been explored to deliver small molecules therapeutics. Hence, they offer significant scope to deliver highly efficacious protein-based drugs through novel and smart drug delivery systems. In recent years several nanotechnology-based drug delivery systems (called as nanomedicines) have been explored to prevent and reverse diabetic side effects. Nanoparticles offer numerous advantages in overcoming the challenges associated with treatments with drugs alone or, even with classic delivery systems such as low solubility in solvents, need of high doses to exhibit therapeutic effect, high toxicity, reduced half-life, aggregation or, enzymatic and chemical degradation. Drug loaded nanoparticles are able to overcome these limitations. In addition to this they are able to offer targeted delivery to specific cells or tissues, sustained delivery of both water insoluble drugs as well as

macromolecules and minimize side effects [8,9]. This article covers various approaches that have been explored to deliver drugs to the posterior segment of eye to treat AMD and DR. The challenges associated with existing drug delivery systems and recent advances in intraocular and nanoparticle-based delivery systems are also discussed.

2. Pathophysiology of DR

Several factors such as hyperglycaemia, hyperlipidemia, hypertension, metabolic dysfunction, and ischemia either alone or in combination with other comorbidities can contribute to the development of DR. These diseases are known to play key roles in the pathogenesis of DR but the exact underlying biochemical mechanisms remain incompletely understood [10]. In fact, multiple signaling pathways are activated in above mentioned conditions and are involved in the pathogenesis of DR. The components that get upregulated include polyol pathway, Protein Kinase C (PKC) pathway, oxidative stress, Advanced Glycation End (AGE) products formation and accumulation, hexosamine pathway, Rennin-Angiotensin-Angiotensinogen System (RAAS), Vascular Endothelial Growth Factors (VEGF) and inflammatory factors [11-13]. These, in turn, cause molecular, structural and functional alterations in the diabetic retina (Fig. 1).

During hyperglycemic condition, polyol pathway is activated by converting glucose into sorbitol in the presence of co-factor nicotinamide adenine dinucleotide phosphate (NADPH), a cofactor. As this cofactor is not available to maintain the activity of glutathione reductase (GR), therefore GR becomes unavailable to maintain the level of reduced glutathione (GSH). Deficiency of GSH, in turn, enhance the levels of reactive oxygen species (ROS) leading to oxidative stress. This oxidative stress further contributes to structural and functional alterations in the retinal microvasculature [10,14-16]. A major manifestation of DR is the microvascular impairment and compromised blood retinal barrier permeability. Increased VEGF levels in response to hypoxia and vascular filtrate may lead to neovascularization and retinal tissue damage in patients as well as in rodent models of the disease. This breakdown of blood retinal barrier may also aggravate the inflammatory cascades in the DR retina by activation of P2X7 receptor leading to pericyte cell death [17,18]. Human vitreous samples obtained from DR patients indeed revealed enhanced pro-inflammatory IL1β and IFNγ levels along with increased VEGF levels [19]. Further studies may be re-

quired to establish the time-course of these molecular changes in the retina which in turn may help to develop mechanism-based therapeutics.

In addition, activation of the PKC pathway in hyperglycemic condition leads to decrease in blood flow, increase in vascular permeability, and eventually retinal neovascularization due to induction of growth factors such as VEGF [20]. RNA-binding proteins (RBPs) regulates VEGF expression and in DR human antigen R (HuR), RBPs, expression is upregulated, augmenting in abnormal increase of VEGF in the diabetic retina [21]. Moreover, in hyperglycemic conditions, AGE formation takes place as a by-product due to reaction of glucose and/or other carbohydrates with proteins, amino acids, nucleic acid and lipids. AGE accumulation in the basement membranes of retinal cells alters the structure of retinal capillaries [22,23].

As the biochemical reactions are altered, for example in case of deficient glycolysis reactions, there is excess amount of intracellular glucose and accumulated glucosamine activates the hexosamine pathway. These biochemical alterations are responsible for changes in protein functions, reduce cell protection mechanism and create an environment of apoptosis in the retinal neurons [24]. Another reason for such alterations is the upregulation of RAAS. RAAS is composed of three major components: renin, angiotensin II, and aldosterone. It increases the VEGF level and leads to abnormal retinal angiogenesis and progression of retinopathy [25,26]. Thus, in-depth molecular level understanding of contributing factors and biochemical mechanisms will help in greater understanding of the disease progression and further advances in novel formulation design for the overall management of DR [8,27].

There are mounting evidence suggests DR pathogenesis is related to neurodegeneration of the retina [28,29]. Studies have shown that under hyperglycemia conditions retinal ganglion cells (RGCs) undergoes apoptosis due to shortage of neurotrophic factor particularly brain derived neurotrophic factor (BDNF) [30]. Serum of DR patients and animal models showed significant decreased in level of BDNF [31]. Tropomyosin-related kinase B (TrkB) receptor are well known to expressed in the retina and BDNF has high affinity for TrkB [32-34]. Liu et al have recently demonstrated the neuroprotective role of BDNF through TrkB/ERK/MAP pathway in the hyperglycemic condition in culture retinal neurons cells [30].

3. Currently available drugs and their delivery systems

3.1.1 Steroidal anti-inflammatory drugs

Corticosteroids are a class of drugs with high anti-inflammatory activity. These drugs are capable of reducing vascular permeability and the blood-retinal barrier (BRB) breakdown, to down-regulate VEGF expression and/or production, and the capacity to inhibit matrix metalloproteinases [35]. The administration of corticosteroids in the management of DR includes the peribulbar, sub-tenon and intravitreal injections. Cortiject implant (NOVA63035; Novagali Pharma) is a tissue-activated protective corticosteroid prodrug available as a preservative- and solvent-free emulsion. A single emulsion injection provides the therapeutic action for about six to nine months' time period [35]. Triamcinolone acetonide (TA) is the most common corticosteroid used in the management of DR and is administered intravitreally. It is important to add here that intravitreal route is the most efficient approach to deliver the drug to the posterior segment of the eye [36]. In situ-forming poly (lactic-co-glycolic acid) (PLGA) implants have been used to achieve release of TA for 42 days [3,37]. In one of the studies, TA loaded photo crosslinked poly (ethylene glycol) diacrylate (PEG-DA) implants showed release of drug upto 28 days [38]. TA is available in the form of injectable suspension (80 mg/mL) as Trivaris® for intravitreal use. Each syringe of the sterile aqueous gel suspension contains 8 mg triamcinolone acetonide in 0.1 mL (8% suspension) in a vehicle containing w/w percents of 2.3% sodium hyaluronate; 0.63% sodium chloride; 0.3% sodium phosphate, dibasic; 0.04% sodium phosphate, monobasic; and water for injection. Trivaris® is preservative-free with a pH of 7.0 to 7.4 [39]. Another preparation of TA is Trisence® suspension that is packaged as a single-use 1 mL vial, at a concentration of 40 mg/mL. It is also a preservative free formulation [39].

Surmodics, Inc. (Irvine, CA) has developed the TA loaded I-vation Intravitreal Implant with sustained release of drug to provide controlled long-term drug delivery into the posterior chamber of the eye [40]. The I-vation Intravitreal Implant is comprised of a nonferrous metal alloy, MP35N. The narrow wire diameter of the implant allows for minimally invasive placement through a 25-g to 30-g needlestick. The unique helical shape of the device maximizes the surface area available for the drug-eluting portion

of the implant and enables secure, suture less anchoring of the device against the surface of the sclera. The thin scleral cap resides under the conjunctival membrane to minimize foreign-body sensation and the design parameters allow the implant to remain outside the visual field and safely away from the lens.

Fluocinolone acetonide (FA) is another steroid commonly used in the form of non-biodegradable implants (Retisert®) for the treatment of DR [41]. Retisert® is commonly used as the first generation of ocular drug delivery implants. It is comprised with drug tablets coated with non-biodegradable polymers such as polyvinyl alcohol (PVA), ethylene-vinyl acetate (EVA), and silicones. In Retisert®, the drug tablet is coated with a non-permeable silicone featuring an orifice to allow drug release. A permeable PVA membrane is kept between the silicone orifice and the tablet to control the rate of diffusion through the orifice [3,42]. It is reported to release the drug for 2.5 years. Other nonbiodegradable implants loaded with FA in the market is Iluvien® (Alimera Sciences, Inc). It is comprised of a polyimide tube loaded with a drug-PVA matrix, and capped on one end with a silicone adhesive [3]. It is reported to release the drug for 3 years. Similarly, Yutiq® (EyePoint Pharmaceuticals, Watertown, MA), is a polyimide nonbioerodible intravitreal micro-insert releasing FA over 36 months.

Dexamethasone is used in the treatment of DR as it inhibits leukostasis, enhances the barrier function of tight junctions, and inhibits the release of local inflammatory factors. Its anti-inflammatory activity is six-folds greater than that of triamcinolone and 30-folds greater than cortisol**.** The marketed implants containing dexamethasone are currently being used include Ozurdex® and Surodex® [35]. Most of these steroids are delivered through intravitreal injection/implant and are reported to lead to side effects such as cataract, glaucoma, retinal detachment, vitreous hemorrhage, and endophthalmitis [35]. Ozurdex® is the first sustained-release biodegradable steroid implant that was developed by Allergan and approved in 2009 for the treatment of macular edema following vein occlusion and noninfectious posterior uveitis. The intravitreal implant comprises PLGA co-extruded with dexamethasone (NOVADUR). It is placed in the vitreous through the pars plana by needle injection and releases peak doses of dexamethasone for 2 months followed by a lower dose for an additional 4 months [43]. Another dexamethasone loaded intraocular implant that got approved in 2018 is DEXYCU® that delivers the drug to the posterior segment of eye and provides release of drug upto three weeks [3]. Surodex (Allergan, Inc., Irvine, CA, USA) is a rod-shaped biodegradable matrix implant consisting of dexame-

thasone and poly lactide-co-glycolide acid (PLGA) with hydroxypropyl methylcellulose (HPMC). It provides sustained drug release at a constant rate of 60 µg over 7–10 days [44]. The NMP (N-methyl-2-pyrrolidone) based in situ-forming PLGA implants have been used to release TA [37] and dexamethasone [45]. The release was sustained upto 42 days for TA and 1 week for dexamethasone.

Bucolo et al (2018) conducted a systematic review to evaluate the long-term safety of repeated dexamethasone intravitreal implant (DEXi) 0.7 mg for management of diabetic macular edema. The retreatment of DEXi was considered on a pro re nata (PRN) basis at any time or starting from month three or four. About 1/3 of the eyes were retreated before six months from first injection (range 0e86.7%). Mean retreatment average time was 5.3 ± 0.9 months, with an estimated average of 1.3 injections each six months. The study revealed that repeated DEXi administration showed an acceptable long-term efficacy/safety ratio [46].

3.1.2 Non-steroidal Anti-inflammatory drugs

Intraocular inflammation is the second mechanism for the DR development. For this reason, non-steroidal anti-inflammatory drugs (NSAIDS) are used to inhibit production of prostaglandins. These NSAIDS protect the diabetic retina from vascular damage and retinal microangiopathy. The NSAIDs that are used for topical application include salicylic acid, indole acetic acid, aryl acetic acid, aryl propionic acid and enolic acid derivatives. The major challenges associated with delivery of NSAIDs is their weakly acidic nature. The drugs get ionized at the pH of the lachrymal fluid. Due to ionization, their permeability through the anionic cornea gets decreased. Hence, reducing the pH of the formulation increases the unionized fraction of the drug which enhances permeation. However, being acidic, NSAIDs are inherently irritant and reducing the pH of formulation further increases their irritation potential, as well as decreasing their aqueous solubility. Moreover, due to anionic nature of NSAIDs, their formulation development with cationic quaternary ammonium preservatives leads to the formation of insoluble complexes [47,48]. The studies on streptozotocin induced diabetic rat model, aspirin was found to decrease the adhesion of leukocytes to the retina and prevented capillary apoptosis and thus leading to retinal protection from streptozotocin induced DR in rat retina [49]. In another studies, nepafenac COX-1 and COX-2 inhibitor, impedes diabetes-related abnormalities with no side effects on

neuron degeneration [50]. Meloxicam, COX-2 inhibitor, demonstrated inhibition of retinal capillary loss in experimental model of diabetic rats [51].

3.1.3 Antiangiogenic (Anti-VEGF) intravitreal injections

Pegaptanib, Bevacizumab (BVZ), Ranibizumab (RBZ), and Aflibercept (AFB) are being currently used in the management of DR to slow down disease progression and reduce the risk of vision loss. Anti-VEGF drug molecules play a critical role in DR's treatment by preventing blindness and improving vision in diabetic patients [52]. Pegaptanib (first antiangiogenic agent) is highly effective in treatment of DR. Pegaptanib is a polynucleotide aptamer that is designed to target the 165 isoform of VEGF-A. It aids neovascular age-related macular degeneration by binding to VEGF which in order reduces angiogenesis and vessel permeability. Pegaptanib is used sporadically in clinical practice. In VISION-1 (VEGF Inhibition Study in Ocular Neovascularization-1) showed that the intravitreal injection at a dose of 0.3 mg pegaptanib every 6 weeks for 1 year showed half the vision loss in subjects as compared to those who received Sham. It has shorter half-life, due to which its effect persists only for a short time [53]. BVZ (Avastin; Genentech) is a recombinant humanized monoclonal IgG1 antibody, and inhibits angiogenesis by binding and neutralizing VEGF-A. BVZ is injected through intravitreal route. The studies showed that BVZ inhibits the proliferation of blood vessels in the eye by blocking VEGF [54]. It should be noted that BVZ is used off-label because it is not approved for eye diseases. In one of the studies, BVZ loaded chitosan nanoparticles embedded in a matrix of hyaluronic acid and zinc sulfate have been explored to provide long-term sustained release of BVZ [55]. This system was found to enhance sustained release of BVZ as compared with BVZ particles. In another study porous polydimethylsiloxane/polyvinyl alcohol composite drug delivery system has been used to deliver infliximab for 3 months in rabbits after an ocular burn [56]. RBZ **(**Lucentis; Genentech, South San Francisco, California**)** is a humanized IgG1 monoclonal antibody fragment created from the same parent mouse antibody as bevacizumab against VEGF-A. The mechanism of RBZ is similar to that of BVZ but it showed better anti-neovascularization in DR when compared to laser therapy treatment [57]. AFB (Eylea; Regeneron, Tarrytown, New York) is a soluble decoy receptor that binds VEGF-A, VEGF-B and placental growth factor (PIGF) with a greater affinity than the body's native receptors. It is called a decoy receptor as VEGF does not bind to its

original receptors and mistakenly binds with aflibercept, thereby reducing VEGF's activity. AFB has been reported to exhibit the highest affinity for VEGF among all anti‐VEGF drugs, almost 100‐folds as compared to BVZ or RBZ. The use of anti-VEGF drugs can cause serious complications in the eyes like impaired wound healing, retinal detachment, endophthalmitis, hypertension, proteinuria, and increased risk of CVS diseases [58]. Other major challenges associated with anti-VEGF agents is their high cost of care.

Monthly (treatment every 4 weeks) and bimonthly (treatment every 8 weeks after 3-month loading) fixed regimens were used in the registration trials for RBZ and AFB, respectively. Number of clinical trials have been carried out to explore the therapeutic potential of RBZ alone or in combination with AFB or BVZ against AMD/DR. These include *MARINA* (Minimally classic/occult trial of the Anti-VEGF antibody RBZ in the treatment of Neovascular AMD), *ANCHOR* (ANti-VEGF Antibody for the Treatment of Predominantly Classic CHORoidal Neovascularization in AMD), The VEGF Trap Eye: Investigation of Efficacy and Safety in Wet AMD studies (*VIEW 1* and *VIEW 2*), The *SUSTAIN* (Study of RBZ in Patients with Subfoveal Choroidal Neovascularization Secondary to Age-Related Macular Degeneration), *HARBOR, CATT* (Comparison of Age-Related Macular Degeneration Treatments Trials), *IVAN* (Inhibition of VEGF in Age-related choroidal Neovascularisation), *PIER* (Phase IIIb, multicenter, randomized, double-masked, sham Injection–controlled study of the Efficacy and safety of RBZ), *EXCITE* (Efficacy and Safety of RBZ in subjects with Subfoveal and CNV secondary to AMD), *LUCAS* (Lucentis Compared to Avastin Study), *TREX-AMD* (Treat and Extend Protocol in Patients with Wet Age-Related Macular Degeneration) and *TREND* (TReat and extEND) [59]. The dose explored for RBZ were 0.2 mg, 0.3 mg, 0.5 mg and 2 mg either on monthly, bimonthly or, quarterly basis. The dose explored for AFB was 0.5 mg and 2 mg. For BVZ, it was 1.25 mg.

In October 2019, FDA has approved brolucizumab (Novartis, Basel, Switzerland). Brolucizumab is a 26 kDa, humanised single-chain antibody fragment that, like existing anti-VEGF therapy, targets VEGF-A. Two 96-week, double-blind, multicentre, phase III clinical trials named as *HAWK* (Efficacy and Safety of RTH258 versus Aflibercept Study 1) and *HARRIER* (Efficacy and Safety of RTH258 versus Aflibercept Study 2) were conducted to investigate the potential of brolucizumab 6 mg (HAWK and HARRIER) and brolucizumab 3 mg (HAWK) versus aflibercept 2 mg in 1817 subjects with untreated nAMD. In these studies, there was a 3-month loading

phase, followed by dosing every 12 weeks for the brolucizumab groups, with an option to decrease to 8-week dosing based on evidence of disease activity; aflibercept was dosed bimonthly per its label. At 2 years, brolucizumab demonstrated non-inferiority in BCVA compared with aflibercept with a similar safety profile. There was an improvement of 6.6 and 6.1 early treatment of diabetic retinopathy (ETDRS) letters with brolucizumab 6 mg and 3 mg, respectively, vs 6.8 ETDRS letters with aflibercept in HAWK ($p<0.001$) and 6.9 ETDRS letters with brolucizumab 6 mg vs 7.6 ETDRS letters with aflibercept in HARRIER ($p<0.001$). More than half of brolucizumab 6 mg eyes were maintained on dosing every 12 weeks through 48 weeks.29 Based on these two trials, brolucizumab may have similar efficacy compared with aflibercept while providing some relief in injection treatment frequency [59,60]. Abicipar pegol (Allergan, Dublin, Ireland) is a DARPin-based (designed ankyrin repeat protein) anti-VEGF therapeutics that is derived from natural ankyrin repeat proteins, which bind to a single target. The *CEDAR* (Safety and Efficacy of Abicipar Pegol in Patients with Neovascular AMD NCT02462928) and *SEQUOIA* (Safety and Efficacy of Abicipar Pegol in patients with Neovascular AMD NCT02462486) trials are 96-week, phase III, multicentre, randomised control trials comparing three different groups (n=817, n=831): abicipar pegol 2 mg monthly for the first 3 months, followed by injections every 8 weeks (n=265, n=267); abicipar pegol 2 mg on day 1, week 4 and week 12, followed by injections every 12 weeks (n=262, n=265); and ranibizumab 0.5 mg every 4 weeks (n=290, n=299) [59,61]. The primary endpoint at 1 year was non-inferiority to ranibizumab, which was met. The mean change in best corrected visual activity (BCVA) for the three groups was 6.7, 5.6 and 8.5 in CEDAR, respectively, while the mean change in BCVA was 8.3, 7.3 and 8.3 in SEQUOIA, respectively [59].

3.1.4 Vitreous agents

Vitrase (hyaluronidase ovine, ISTA Pharmaceuticals Inc.) is the first and only pure, preservative-free ovine hyaluronidase that is being used for the management of vitreous hemorrhage without any reported serious adverse event in the treated eye [62]. Intravitreal injections of other pharmacological agents like plasmin and microplasmin are reported to treat diabetic macular edema (DME) and PDR by inducing posterior vitreous detachment and reducing retinal neovascularization [63]. Micro-

plasmin improved the vitreomacular adhesion, cured full thickness macular hole and vision impairments in patient-reported visual function [64].

3.1.6 Antihyperlipidimics

3.1.6.1 Fibrates

These are lipid lowering drugs used for the treatment of dyslipidemia. Fenofibrate is used for reducing the total cholesterol, low density lipoproteins (LDL). It acts against DR via activating PPAR-α that causes inhibition of inflammation by suppression of nuclear factor κB and by direct binding to genes encoding proinflammatory cytokines. Activation of PPAR-α mediated by fenofibrate also leads to inhibition of VEGFR2 expression and neovascularization in human umbilical endothelial cells [65].

In a placebo controlled Fenofibrate Intervention and Event Lowering in Diabetes (FIELD) trial conducted on 9795 patients suffering from T2DM for 5 years fenofibrate's treatment at a dose of 200 mg/day, reduced the risk of DR's progression rate by 79% as compared with placebo [66,67]. In Action to Control Cardiovascular Risk in Diabetes (ACCORD) Eye study conducted on 2856 patients with T2DM for 4 years fenofibrate's treatment at a dose of 160 mg/day, reduced DR's progression rate by 40% as compared with placebo [68].

3.1.6.2 Statins

Statins are reported to inhibit 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase and mevalonate pathway that results in reduction of low-density lipoprotein cholesterol. This further results in decreased production of isoprenoids, geranylgeranyl pyrophosphate and farnesyl pyrophosphate [69]. This causes reduction in addition of farnesyl and geranylgeranyl to cysteine residues of proteins, which modulates a host of pathogenetic mechanisms involved in DR, including inflammation, angiogenesis, oxidative stress, and endothelial dysfunction [69,70]. Statins are also reported to cause antiangiogenic actions by suppression of VEGF phosphorylation in retinal endothelial cells [71]. Statins reduce the expression of matrix metalloproteinases (MMP) in retinal pigment epithelial cells. Thus they prevent the breakdown of the

BRB[72]. In some of the preclinical studies statin induced endothelium-dependent and nitric oxide-mediated vasodilation in retinal arteries has also been observed [73]. In animal models of DR, treatment with statins has been reported to prevent the up-regulation of VEGF and preservation of the BRB through their antioxidant [74-76] and anti-inflammatory effects[77,78].

Somatostatin is one of the first reported drugs that has been used topically as eye drops for the treatment of DR. It can cross the BRB and reach the posterior region of the eye and can show its pharmacological effect by preventing inflammation and vascular leakage [79]. Simvastatin (20 mg/day) is reported to retard the progression of DR in a randomized clinical trial conducted for 6 months on 50 patients with DM [45]. In two of the clinical studies, treatment with atorvastatin to 18 patients for 12 months at a dose of 20 mg/day and 30 patients for 4.5 months at a dose of 10 mg/day, led to reduction in formation of hard exudates and fluorescein leakage [80,81].

3.1.7 Antihypertensive agents

Hypertension is reported as one of the contributing factors for the development of DR, as proper control on blood pressure (BP) helps in reducing the probability of development of DR in both type 1 and 2 diabetic patients. Angiotensin-II Converting Enzyme (ACE) inhibitors are the most commonly used drugs to treat hypertension in diabetic patients because the RAAS is also expressed in diabetic retina [82]. Blocking of RAAS using either ACE inhibitor (enalapril) or angiotensin receptor blocker (ARB) (losartan) helps in reducing DR's progression. ARB inhibits the binding of angiotensin II to AT1 receptor while RAAS inhibit conversion of angiotensin I to angiotensin II. About 65% to 70% reduction in the rate of DR's progression has been observed after treatment with ARB agents such as enalapril and losartan respectively [83]. Candesartan is an another example of ARB that is used for the treatment of mild DR [84,85]. ARBs are preferred for those patients who have reported adverse reactions with use of ACE inhibitors. RAAS blockers inhibit the activities of ACE, rennin, and type I and type II angiotensin. Thus, they help in maintaining BP and fluid balance in the body by reducing the levels of prorenin, renin, and Angiotensin-II (AT-II) in the vitreous humor of patients with DR. RAAS inhibitors can also decrease the level of retinal VEGF in diabetic patients having DR. Valsartan *(*an AT-II receptor an-

tagonist) is one of the RAAS inhibitors that stops the increase in VEGF levels in the retina [82]. Candesartan blocks the activity of AT-I and reduces the progress of DR. Other inhibitors of AT-I (e.g., losartan and candesartan cilexetil) are undergoing clinical trials for the treatment or prevention of DR [85].

3.1.8 Antiplatelet agents

Platelet activation is one of the side effects of chronic hyperglycemia in addition to retinal-inflammation, aggregation, and thromboxane A2 accumulation. Aspirin is an antiplatelet agent. It is used to slow down the progression of DR when used in combination with dipyridamole [86]. Lisinopril is another antiplatelet agent that helps in reducing the progression of DR in diabetic people [87].

3.1.9 Protein kinase C (PKC) inhibitors

PKC regulates angiogenesis, blood flow, and cell permeability in the eye. PKC activity increases because of oxidative stress on vascular endothelial cells which can be inhibited by PKC inhibitors [88]. Pazopanib is a selective inhibitor of glycation that leads to the inhibition of VEGF [89]. Ruboxistaurin (RBX*)* also inhibits the PKC 1 and 2 receptor activity and helps in the prevention of DR. It is a well-tolerated drug and helps in delaying the time of vision loss [90].

3.1.10 Growth hormone (GH) inhibitors

The effect of growth hormone (GH) in the pathogenesis of DR was first reported by clinical observation of regression of severe PDR in 1953 [91]. Somatostatin, is a growth hormone inhibiting hormone (GHIH) that regulates the endocrine system. Role of several synthetic analogues of somatostatin in the management of DR has been explored and found to be directly linked with the inhibition of angiogenesis through somatostatin receptors present in endothelial cells [92]. Octreotide, a somatostatin analogue, is being used for the management of DR. The use of octreotide and other somatostatin analogues seems to regulate angiogenic responses to the retinal hypoxic environment through a modulation of retinal levels of VEGF and its receptors [93].

3.1.11 Carbonic anhydrase inhibitors

Carbonic anhydrase is a metalloenzyme that converts carbon dioxide and water to bicarbonate and protons. It plays a significant role in acid base balance. Retinal pigment epithelium contains membrane bound carbonic anhydrase. Carbonic anhydrase is present inside the retinal muller cells and red/green cones (not rods). To maintain the pH gradient, carbonic anhydrase is created by metabolic activity of the cells. Elevated level of carbonic anhydrase was observed in vitreous of patients with PDR. The carbonic anhydrase inhibitors such as acetazolamide, dorzolamide, benzolamide, on oral administration, downregulate the progression of DR, thus preventing the vision loss. Some oral carbonic anhydrase inhibitors such as brinzolamide, methazolamide, ethoxzolamide, butazolamide, dichlorphenamide, flumethiazide can also be used in the treatment of DR [94].

3.1.12. Plant based agents

Drugs from plant origin are also used for the treatment of diabetes and its complications including DR from very old time. The herbal medicines are beneficial in DR as they are considered safe, with no or less side effects, decrease elevated blood glucose level and provide protective effect on retina. Some natural aldose reductase inhibitors (ARIs), derived from plant sources are *Ganoderma lucidum, Tinospora cordifolia,* and *Ocimum sanctum. Ganoderma lucidum* protects retina from oxidative damage [95]. *Tinospora cordifolia* exhibits its protective action by preventing retinal oxidative stress (ROS) produced by pro-angiogenic overexpression and pro-inflammatory mediators [96]. *Ocimum sanctum* exerts its protective effect against DR when used in combination with vitamin E [97]. Curcumin, isolated from *Curcuma longa*, helps in treating DR by inhibiting VEGF and thus having anti-VEGF action [98]. It also shows antioxidant and anti-inflammatory effects in the experimental rat retina [99]. In one of the studies, the effects of curcumin on human retinal pigment epithelial (RPE) cells exposed to high glucose (HG) insult was performed on RPE cells that were cultured both in normal and HG conditions to assess the effects of curcumin on the cell viability, nuclear factor erythroid 2-related factor 2 (Nrf2) expression, HO-1 activity, and extracellular-signal-regulated kinase (ERK) 1/2 expression. RPE cells exposed to HG insult were treated with curcumin. The obtained results indicated that treatment with curcumin caused a significant decrease in terms of apoptosis. It was also able to induce HO-1 expression via Nrf2 activation and counteracted the damage elicited by HG. Hence it was concluded that curcumin provided protection

against HG-induced damage in RPE cells through the activation of Nrf2/HO-1 signalling that involved the ERK pathway [100]. In another study, self-assembling aqueous ocular nanomicellar eye drop loaded with curcumin were formulated using quality by design approach to treat age-related macular degeneration (AMD) in eye. The results indicated that curcumin loaded nanoformulation exhibited significant protection of retinal (D407) cells against H2O2-induced oxidative stress. In addition to that the study conducted using ELISA indicated significant reduction in VEGF release in D407 cell lines by this formulation, which is an indication of reduction in risk of angiogenesis. *In vitro* drug release kinetics suggested a sustained drug release profile indicating a long-term protection ability of the developed formulation against oxidative stress to retinal cells [101].

Pycnogenol, extracted from the bark of the maritime pine tree demonstrated its protective effect by providing antioxidant and anti-inflammatory activity in DR [102]. Genistein was found to be effective in prevention of retinal vascular leakage in a rat model [103]. Green tea also reduces progress of DR due to its anti-oxidant properties [104]. Hesperetin, quercetin and rosmarinic acid have also exhibited reduction in DR by preventing angiogenesis via antioxidant activity [105].

The plant based dietary supplements such as flavonoids, carotenoids, vitamin A, C (ascorbic acid), and E (tocopherols) possess strong antioxidant effect and prevent lipid peroxidation in the body system. Resveratrol has shown protection against oxidative stress in retinal pigment epithelial cells [106]. The use of α-tocopherol helps in prevention of diabetes-induced abnormal retinal blood flow [8]. Various agents that are used to treat DR are summarized in Table 1.

The major reasons for the moderate success of existing therapies include protein binding, reduction in drug's concentration on instillation, less space at ocular site, invasive process, and cost of treatment [88]. These factors are discussed below in detail:

- **Protein binding**- Drug binding with proteins present in tear fluid leads to decrease in the absorption of the drug.
- **Reduction in drug's concentration**- There is a decrease in the drug concentration of drug on administration due to various defense mechanisms including tear formation, blinking, and flow of the material *via* nasolacrimal duct. Metab-

olism and enzymolysis of the drugs also occur due to the presence of enzymes in the eye.

- **Less space**- Limited capacity of conjunctival sac, which is approximately 30□µL without blinking. This often leads to spillage of the drug upon administration.
- **Anatomical barriers**- Complex anatomical structure of the eye, little transparency of the cornea, low absorptive surface, and lipophilicity of corneal epithelium are the major reasons for the limited success of conventional dosages forms in several ocular diseases when administered through ocular route.
- **Expensive**- The intravitreal injections are expensive. For example, Ranibizumab (Lucentis) costs approximately $2,150 per dose, pegaptanib sodium costs $1,000 per cost, bevacizumab (Avastin) $55 per dose and aflibercept (Eylea) costs €943 per injection [107].

Table 1. Drugs explored to manage DR

Category	Mechanism of action	Example of drugs	Marketed formulations	Dosage form	Adverse effects	Reference
Anti-Inflammatory Steroids (Corticosteroids)	Anti-inflammatory and anti-angiogenesis activities by modulating various proinflammatory mediators, such as tumor necrosis factor-alpha (TNF-α), interleukin-1β (IL-1β), and VEGF	1.Triamcinolone acetonide	1. Triesence® (Alcon pharmaceuticals, Geneva, Switzerland) 2. Trivaris® (Allergen, Irvine, CA, USA)	Intravitreal Injection	Cataract, glaucoma and elevation of intraocular pressure Retinal detachment, vitreous hemorrhage, and endophthalmitis	[36]
		2. Sustained-release triamcinolone acetonide	I-vation® (Surmodics, Inc., USA)	Biodegradable intravitreal implant	Elevation of intraocular pressure	[35]
		3. Sustained release fluocino-	1. Iluvien® (Alimera Sciences Inc.,	Non-biodegrada-	Cataract, glaucoma	[41]

		lone acetonide	Alpharetta, GA, USA) 2. Retisert® (Bausch & Lomb Inc., Rochester, New York, USA)	ble intravitreal insert	and elevation of intraocular pressure	
		4. Sustained release Dexamethasone	1. Ozurdex (Allergen, Irvine, CA, USA) 2. Posurdex®, (Allergen, Irvine, CA, USA)	Biodegradable intravitreal implant	Elevation of intraocular pressure	[35]
		6. Tissue-activated proprietary corticosteroid prodrug	Cortiject implant (NOVA63035; Novagali Pharma)	Intravitreal implant in the form of emulsion	Not reported	[35]
		7. VerisomeTM liquid delivery system based sustained re-	IBI-20089 (Icon Bioscience Inc., Newark, Calif.)	Intravitreal injection	Not reported	[35]

		lease triamcino-lone acetonide				
NSAIDs	COX-1 and COX-2 inhibitors	Nepafenac	Nevanac® (Alcon pharmaceuticals, Geneva, Switzerland)	Topical	Not reported	[27]
	COX-2 inhibitor	Meloxicam	Mobic® (OEM manufacturers India)	Oral	Blurred vision, conjunctivitis, optic neuritis	[27]
	Anti-inflammatory by modulating TNF-α	Etanercept	Enbrel® (Amgen Inc., and Wyeth, Germany)	Intravitreal injection	Not reported	[27]
	Anti-inflammatory by modulating TNF-α	Infliximab	Remicade® (Janssen Biotech, Inc., Philadelphia, USA)	Intravitreal injection	Not reported	[27]
Antiangiogenic agents	Primary targets the subfamily protein, VEGF, in which its over-expression plays a crucial role in the pro-	1. Bevacizumab	Avastin® (Genentech, South San Francisco, CA)	Intravitreal injection	Retinal detachment, Endophthalmitis, Impaired wound healing, Hypertension,	[54]

	gression of DR and AMD				Proteinuria	
		2. Ranibizumab	Lucentis® (Genentech, South San Francisco, CA)	Intravitreal injection	Retinal detachment, Endophthalmitis, Impaired wound healing, Hypertension, Proteinuria	[57]
		3. Aflibercept	EYLEA® (Regeneron Pharmaceuticals Inc., and Bayer HealthCare Pharmaceuticals, New York, USA)	Intravitreal injection	Retinal detachment, Endophthalmitis, Impaired wound healing, Hypertension, Proteinuria	[58]
		4. Pegaptanib	Macugen® (Bausch and Lomb, USA)	Intravitreal injection	Retinal detachment, Endophthalmitis,	[58]

					Impaired wound healing, Hypertension, Proteinuria	
		5. Integrin alpha5beta1 antagonist	JSM6427 (under clinical trial, Jerini AG, Germany)	Intraocular implanted osmotic pump that releases drug for six months	Not reported	[108]
		6. Ubiquitin-like-conjugating enzyme	ATG3 (under clinical trial by CoMentis Inc., USA)	Topical eye drop	Not reported	[108]
		7. Pazopanib	Votrient® (Glaxo SmithKline, USA)	Oral	Not reported	[108]
Vitreous Agents	Reported to treat DME and PDR by inducing posterior vitreous detach-	1. Vitrase	Hyaluronidase ovine (ISTA Pharmaceuticals Inc., Irvine, CA)	Intravitreal injection	Retinal detachment, Endophthalmitis, Impaired wound	[62]

	ment				healing, Hypertension, Proteinuria	
		2. Microplasmin	Ocriplasmin® (Thrombo-Genics NV, Belgium)	Intravitreal injection	Retinal detachment, Endophthalmitis, Impaired wound healing, Hypertension, Proteinuria	[63]
Systemic agents (Hypolipidemic Agents)	1. Fibrates They act via non-lipidemic mechanisms, mainly activating PPAR-α for DR prevention. Activation of PPAR-α mediated by fenofibrate led	Fenofibrate	Tricor® (AbbVie) Lipofen (Kowa Pharmaceuticals America Inc) Lofibra® (**Teva**), Lipanthyl, Lipidil, Lipantil micro and Supralip (**Abbott Laboratories**) Fenocor-67 (Ordain Health Care) Fibractiv 105/35 (Co-	Oral	Blood clot, Headache, back pain, Nausea	[65]

	to inhibition of VEGFR2 expression and neovascularization in human umbilical endothelial cells		gentrix Pharma, India), Fenogal (SMB Laboratories) Antara (Oscient Pharmaceuticals) Stanlip (Ranbaxy, India)			
	2. Statins These are used to treat hyperlipidemia by mainly lowering the triglyceride levels, total and low-density lipoprotein (LDL) cholesterol, small LDL cholesterol particles, and apolipoprotein B while in-	1. Simvastatin 2. Rosuvastatin	Zocor® (Merck & Co., Inc. USA)	Oral	First pass metabolism, Less therapeutic effect	[79]

	creasing high-density lipoprotein (HDL) cholesterol.					
Systemic agents (Antihypertensive Agents)	ACE-2 inhibitors	1. Enalapril Maleate	Vasotec® (BTA Pharmaceuticals, Inc. USA)	Oral	Intestinal angioedema,	[83]
		2. Lisinopril		Intravitreal injection	Retinal detachment, Endophthalmitis, Impaired wound healing, Hypertension, Proteinuria	[87]
	RAAS inhibitors [Angiotensin receptor blocker (ARB)]	1. Losartan	Cozaar® (Merck & Co., Inc. USA)	Oral	1-2% drug reaches to the retina of eye. Repeated dose required. Less therapeutic effect	[83]
		2. Candesartan	Atacand® (Astra Zeneca,	Oral	Hypotension,	[85]

			Europe)		Reduction in GFR, Hyper-kalemia, Anaemia	
Antiplatelet agents	Reduction in mi-croaneurysms formation	Dipyridamole + Aspirin	Aggrenox® (Boehringer Ingelheim, Germany)	Oral	Hearing prob-lem, yellowing of eye, (Jaun-dice), Liver problem	[109]
Systemic agents	Treats moderate to severe NPDR by reducing the overactivation of protein kinase C beta, which is involved in the pathogenesis of DR	Ruboxistaurin	Arxxant® (Eli Lilly, Caroli-na, USA)	Oral	First pass ef-fect, Less ther-apeutic effect	[90]
Systemic agents	Somatostatin De-rivatives (rates of progression to	Octreotide	Sandostatin® (Novartis, Switzerland)	Topical	Increase of IOP, cataract and endoph-	[110]

	PDR, vitreous haemorrhage, and the need for vitrectomy in patients with severe NPDR)				thalmitis	
Systemic agents (Carbonic anhydrase inhibitors)	Inhibition of activity of carbonic anhydrase	1. Acetazolamide 2. Dorzolamide 3. Benzolamide 4. Brinzolamide 5. Methazolamide 6. Ethoxzolamide 7. Butazolamide 8. Dichlorphenamide 9. Flumethiazide	Trusopt® (Dorzolamide) (MSD pharmaceuticals, India), Azopt® (Benzolamide, Alcon pharmaceuticals, Geneva, Switzerland)	Oral	Temporary blurred vision, redness of the eye, red eye	[111]
Growth hormone inhibitor (GHI)	It inhibits insulin and glucagon secretion, prevention of retinal neu-	Somatostatin	Somacheck® (Samarth, India)	Eye drops	Gall stone formation, Lachrymal drainage Tear flow	[79]

	rodegeneration				Blinking of the eye Loss of drug content	

4. Novel drug delivery systems

The challenges of the existing conventional dosage forms suggest rethinking on the need for non-invasive, effective and economical delivery systems. In recent years, NDDS have emerged as effective carriers that can deliver the drug successfully to the retina overcoming the challenges associated with conventional and surgical approaches. The two main approaches in improving the ocular bioavailability are by prolonging the contact time on ocular surface and increasing the corneal permeability. The main aim of NDDS is to reduce the cost and frequency of injections, increase therapeutic effect, minimize the side effects, improve patient compliance, and overcome the limitations associated with the conventional dosage forms[27]. However, the major factors that determine the success of ocular drug delivery include optimization of lipophilic-hydrophilic properties of the polymer-drug system, optimizing rates of biodegradation, safety and mucoadhesiveness as well as biocompatibility of polymers used for the preparation of nanoformulations. Judicious selection of polymer plays an important role in the release kinetics of the drug from nanoformulations. The properties that determine ocular bioavailability from a mucoadhesive formulation include polymer's bio-adhesion characteristics, which are affected by its hydration time, swelling properties, molecular weight, and degree of cross linking. The binding of drug depends on the physicochemical properties of the molecule as well as of the nanoparticle polymer. Moreover, the manufacturing process also affect the quality of nanoformulations, specially their binding property. The polymers that have been used in development of ophthalmic formulations are poly(acrylic acid) (PAA), hyaluronic acid, poly(alkyl cyanoacrylates) (PACA), poly(caprolactone) (PCL), poly(lactic-co-glycolic acid) (PLGA), poly (lactic acid) (PLA), chitosan (CS), Eudragit RL100 and RS100, modified polystyrene, albumin and gelatin are also used [112].

Different nanocarriers that are proposed for the management of DR by various researchers are enlisted in Table 2. These nanocarriers are discussed in detail along with the current traditional treatments and advantages of the proposed nanomedicines to target DR.

4.1 Nanoliposomes

Nanoliposomes are small vesicles having spherical shape consisting of single or multiple lipid bilayer formed from natural or synthetic phospholipids having aqueous core in the center [113]. These are used as vehicles for administration of both, lipophilic (loaded in the bilayer) as well as hydrophilic (loaded in the core) drugs. They help the drugs in reaching to their target site because of their smaller size and longer residence time. They prolong the contact time of the drugs onto the ocular surface and increase corneal permeability by crossing the retinal barriers [114].

In one of the studies, rapamycin loaded nanoliposomes demonstrated improved solubility as compared to that of rapamycin alone. Drug was given via topical route and showed transscleral permeation with high drug retention. This suggested topical delivery for management of ocular disease such as DR [115].
In another study, an artificial virus was used to prepare nano liposomes for use in a number of ocular diseases. Protamine-DNA complex was incorporated inside nanoliposomes for efficient delivery of Rpe65 gene in the RPe65 knockout mice retina, for treatment of blindness. The results showed significant improvement in the vision recovery [116]. In additional studies, liposomes of citicoline (a well-known drug to treat glaucoma) have been prepared and explored for the treatment of DR. The citicoline loaded liposomes were employed as a neuroprotective agent for the treatment of early stage of DR. Effect of citocoline liposomes was evaluated when given topically twice a day for 15 days to mice by estimating retinal neurodegeneration. The liposomal formulation of citicoline showed promising neuroprotective effect in diabetic retina as it led to downregulation of synaptophysin. Moreover, it was found to show substantial anti-inflammatory effects [117].
Nanoparticle of timolol, timolol maleate chitosan coated liposomes (TM-CHL) were prepared by ammonium sulfate gradient coupled with pH gradient method. These liposomes enhanced the precorneal residence time, ocular permeation, bioavailability and prolonged the residence time on cornea without any irritation as compared to the commercially available eye drops of TM. Formulation showed an excellent intraocular pressure-lowering effect which can indirectly cure DR and an effective strategy to improve the ocular absorption of water-soluble TM for the treatment of ocular diseases such as glaucoma, DR etc. [118].
In another study, ranibizumab encapsulated liposomes prepared by dehydration rehydration method for sustained release, greater penetration, higher degree of en-

capsulation, penetration into the sclera, and depot effect as compared to invasive intravitreal injections. Four different compositions of lipids were explored including Dipalmitoyl phosphatidyl choline (DPPC), Cholesterol, 1,2-dipalmitoyl-3-trimethylammonium-propane (DPTAP) and dipalmitoyl phosphatidyl glycerol (DPPG). Four different batches were prepared using DPPC alone and DPPC- Cholesterol, DPPC-DPTAP and DPPC-DPPG. The study indicated maximum entrapment efficiency of drug in liposomes prepared using DPPC-DPPG (Fig 2a). DPPC alone was unable to provide good entrapment efficiency (14 % only). Combination of cholesterol with DPPC was able to increase the entrapment efficiency of drugs while addition of positive (DPTAP) and negative (DPPG) phospholipids increased the entrapment efficiency of drug upto 60% (w/w). The *ex vivo* distribution of liposomes (DPPC, DPPC-Chol, DPPC-DPTAP and DPPC-DPPG) was carried out for 96 hours into the porcine sclera. The DPPC liposomes localized preferentially close to the episcleral region, whereas the DPPC-Chol formulation showed higher degree of intrascleral diffusion. Positively charged DPPC-DPTAP formulation was localized preferentially at the episcleral space and showed no appreciable transport into the sclera. On the other hand, negatively charged DPPC-DPPG liposomes showed to some extent episcleral penetration and transport (Fig 2b). The release profile of Ranibizumab from different lipid-based formulation indicated longer release of drug upto 21 days from DPPC-DPPG liposomes, followed by DPPC-DPTAP liposomes (upto 15 days). Complete drug release was observed from DPPC liposomes of Ranibizumab (100 %) and about 86% from DPPC-Cholesterol liposomes (Fig.2c). Similar observations were also noted for *ex vivo* transport study carried out for 7 hours. The transport of bare Ranibizumab was compared with ranibizumab encapsulated in DPPC, DPPC-Chol, DPPC-DPTAP and DPPC-DPPG liposomal formulations. The bare Ranibizumab showed about 52.7% transport of drug. Similar transport pattern was observed for drug encapsulated in DPPC-Cholesterol liposomes. The DPPC alone showed a better sustained release pattern as that of DPPC-Cholesterol and bare Ranibizumab. However, this difference was insignificant ($p > 0.05$). Better sustained drug transport profiles were observed for DPPC-DPTAP and DPPC-DPPG liposomes (Fig.2d). Authors concluded higher encapsulation efficiency and sustained release profile of ranibizumab loaded in DPPC-DPPG liposomes [119].

Liposomes based phosphodiester oligonucleotide [33P] pdT16 and Luciferase genes were administered via intravitreal route (100 µL) in rabbits to treat DR. Their activity

in the cornea, aqueous humor, iris-ciliary body, lens, vitreous body, and retina was measured. They showed the highest transfection efficiency in the ocular tissue and their transfection-mediated luciferase activity peaked at 3 days. Among the ocular tissues, the highest gene expression was observed in the aqueous humor [120,121].

4.1.1 Limitations of nanoliposomes

Due to environmental factors, their stability is less and *in vivo* release of drug from nanoliposomes is complex [122].

4.2 Nanoparticles

Nanoparticles (NPs) are having size range in nanometers which can easily cross different barriers of the body. NPs are categorized into lipid NPs and polymeric NPs. Polymeric NPs are made up of polymers and lipid NPs are made up of mainly three layers- surface layer, shell layer and the core which are composed of lipids, surfactants and co-surfactants. Because of presence of lipids, permeability of the NPs increases which leads to more retention of the drug in the eye for longer period. Lipid NPs are broadly classified as solid lipid nanoparticles (SLNs) and nanostructured lipid carriers (NLCs).

In the recent studies, NPs were prepared by thin film hydration method and administered intravenously to target the choroid for the management of choroidal neovascularization in treatment of DR as shown in Fig.3. After NPs administration, it was converted to a tissue-targeting state by irradiation in the eye (Fig. 3a). Photo-targeted NPs were formulated by self-assembly of a chemically modified poly (ethylene oxide)-poly (D, L-lactic acid) (PEG-PLA) block copolymer (Fig. 3b). Fig.3c showed the synthesis of caged cell penetrating peptide (CPP) functionalized polymer chain. The developed NPs accumulate the drug specifically in the diseased areas of the eye and reduced the size of neovascular lesion. Further, characterization of the prepared NPs was done by using Transmission Electron Microscopy (TEM). The average size of the prepared NPs was found to be 50 nm (Fig. 3d), Fluorescence emission spectra showed a shift in the wavelength for irradiated NPs 465 nm as compared to normal NPs i.e 481 nm (Fig. 3e). 1H NMR spectra showed the structural peaks of the spectra of free CPP and different NPs in D_2O, with the signature phenylalanine proton peaks highlighted in the blue rectangle. NP-CPP is the nanoparticle formed from CPP-PEG-PLA and mPEG-PLA (1:4 weight ratio). Irradiation was done with a 400

nm LED for 1 min at 50mWcm^{-2} (Fig. 3f) and photocleavage of NP-[CPP] in Phosphate Buffer Saline (PBS) (0.5 mg mL^{-1}), determined by HPLC detected at 390 nm absorbance, after continuous irradiation (50mWcm^{-2}, 400 nm) Fig. 3g [118]. The neuroprotective role of NPs in DR and delivering the drug to the retina was explored by Kowluru, et al., in 2001. In this study, NPs functionalized with neuroprotective substances such as glutamate, somatostatin, and coenzyme Q10 etc. were used for protection of diabetic retina for management of DR disease. These biodegradable polymeric NPs have been explored as carriers to target the specific sites in the eye. The reported polymers were biocompatible, non-antigenic and hydrophilic in nature [123].

Another research group focused on preparation of connexin43 mimetic peptide (Cx43MP) NPs by double emulsion solvent evaporation method for the treatment of the light-damaged the rodent eye. The prepared NPs exhibit sustained release of drug for the treatment of retinal injury leading to downregulation in inflammation markers, maintaining the retinal structure and function of the eye as compared to the conventional dose as well as reduction of other ocular complications associated with repeated use of intravitreal injections. The formulations were found to target choroid layer in 30 min post injection, photostable, preventive to photoreceptor loss improvement of light sensitivity, preventing the loss of the photoreceptor as well as inflammatory nature [124].

Amadio et al (2016) formulated two nanosystems viz. solid lipid nanoparticles (SLNs) and liposomes (SUV) loaded with siRNA silencing HuR expression (lipoplexes) for treatment of DR in rats [125]. In this study the lipoplexes were injected into the eye of streptozotocin (STZ)-induced diabetic rats. The levels of retinal HuR and VEGF were detected by Western blot and ELISA. The results demonstrated that retinal HuR and VEGF were significantly increased in STZ-rats and are blunted by HuR siRNA treatment. It was concluded that lipoplexes with a weak positive surface charge and with a 4:1 N/P (cationic lipid nitrogen to siRNA phosphate) ratio exerted a better transfection efficiency, significantly dumping retinal HuR and VEGF levels [125].

4.3 Nanoemulsion

Nanoemulsions are defined as oil in water type emulsion in which small size droplets are formed having lipid core inside and outer lipid membrane along with surfactants. These droplets can penetrate ocular tissue and release drug at the targeted site. Surface modified nanoemulsions using suitable polymer are also able to provide sustained release effect. This system has advantage of having better therapeutic action, with less side effects, and more patient compliance [126]. In recent studies, an *in situ* nano emulsion gel of loteprednol etabonate (ester-based corticosteroid) formulation was discussed that converted to gel after insertion into rabbit's eye. It was used for the treatment of ocular inflammation after cataract surgery [127].

4.4 Nanomicelles

Nanomicelles are self-assembled molecules having polar head in contact with solvent and non-polar tail towards the center. This system is like liposomes except its external layer. In case of liposomes, the external layer is a phospholipid-based bilayer but in nanomicelles the external layer is a monolayer. These micelles possess nano size and demonstrate higher permeation rate of the drugs without causing any irritation to the eyes. These are made up of surfactants (cationic, anionic or ionic) and polymers [128].

In one of the studies, Dexamethasone loaded nanomicelles were evaluated for transscleral iontophoresis following topical application. These nanomicelles were found to reach the retina, mainly through the conjunctival/scleral pathway [129]. In another study, Dexamethasone-encapsulated polymeric nanomicelles formulation design consisted of poly (ethylene glycol)-poly(ε-caprolactone) di-block copolymer. The optimized formulation increased dexamethasone permeability by 2 times across conjunctival cell line and by 2.5 times across the excised rabbit sclera, as compared to dexamethasone suspension [130]. Cyclosporine-A (0.09%) loaded nanomicellar formulation (Cequa®, Sun Pharmaceuticals Inc) has been approved by FDA for treatment of dry eye diseases. It has shown improved rapid onset of action, as early as four weeks and improvement in tear production as compared to Cyclosporine-A emulsion during clinical trials [131].

4.5 Solid lipid nanoparticles (SLNs)

SLNs are spherical particles with average diameter between 10 and 1000 nanometers and provide more surface area, and high drug loading. These formulations consist of solid lipid, surfactant and co-surfactant along with hydrophilic or lipophilic drugs. SLNs are able to achieve the goal of controlled and site-specific drug delivery because of small size, presence of lipids, enhanced stability, excellent biocompatibility, efficient crossing of biological barriers such as retinal barriers and increased bioavailability [122].

Triamcinolone acetonide (TA) was explored for treatment of posterior ocular diseases like uveitis, inflammation, diabetic macular edema and DR. TA-loaded solid lipid nanoparticles (TA-SLNs) and *in situ* gel (TA-SLN-IG) formulations were prepared by hot homogenization and melt emulsification method (Fig 4a). These were delivered tropically into the deeper ocular tissues. The organic phase was prepared by melting glyceryl monostearate (GMS) and Compritol5 888ATO at 80º C and drug was added with continuous stirring. Aqueous phase was prepared by mixing tween 80 and pluronic F-68 as surfactants, glycerin and distilled water. A pre-emulsion was formed by adding aqueous phase to organic phase with sonication. SLNs were obtained by homogenizing this emulsion. Result showed that lipid based nanoparticulate system, after combining with *in situ* gelling agents, proved to be a promising drug delivery platform for the deeper penetration of drugs to ocular tissues for the treatment of DR. Corneal histology studies were performed with prepared SLNs as shown in Fig.4 (b) below [124].

SLNs of myricitrin (plant-derived antioxidant), were prepared by using cold homogenization method and evaluated in STZ induced T2DM mouse model. Prepared SLNs showed antioxidant, antiapoptotic effects, and antidiabetic activity, which protected the diabetic retina from DR. SLNs of myricitrin improved the antioxidant defense, amount of glycogen, and cellular survival in myotube cells exposed to the hyperglycemic condition. Some of these effects were found to be more pronounced in SLNs-administered groups compared to the group with metformin [132].

In another study, SLNs of ciprofloxacin (CIP) were prepared by ultrasonic melt-emulsification method for its controlled release to treat ocular diseases in particular DR. CIP-SLNs were found to possess diameter of 165 to 320 nm along with high entrapment efficiency. Release studies were performed using dialysis bag having molecular weight between 12–14 kDa in phosphate buffer saline. The formulations

showed a sustained release profile along with higher antibacterial activity as compared to CIP *per se*. Accordingly, it was concluded that CIP might be administered to remain in contact with the retina for longer time intervals by loading in a SLNs [133].

SLNs have been shown to exhibit lower transfection level in human retinal pigment epithelial established cell line (ARPE-19) and are useful as non-viral vectors for gene therapy for treatment of retinal disease such as DR [134].

4.6 Dendrimers

Dendrimers are star-like, highly branched, water soluble, macromolecule systems having three components: a central core, an interior dendritic structure (known as branches), and an exterior surface with the functional surface groups [135]. Drug can entrap into dendrimers via hydrogen bonds, hydrophobic interaction, covalent bonds, and ionic interaction. These synthetic polymer macromolecules can provide sustained release of drug in posterior segment of the eye [136].

In one of the studies, polyamidoamine (PAMAM) dendrimers were synthesized and administered in animal eye for the treatment of retinal neuroinflammation. PAMAM dendrimers via topical route in animal models demonstrated structural and function protection in damaged DR retina [137].

Dendrimers and Polyplexes with anti-angiogenic oligonucleotide-1 and plasmid DNA as genes were used via transfection of human retinal pigment epithelium cells upon complexation with the oligonucleotide for the treatment of DR. The level of transfection was indirectly measured by the downregulation of the hVEGF protein (in comparison to the transfection agent cytofectin) [138-140].

4.7 Nanostructured lipid carriers (NLCs)

NLCs are drug delivery systems which are generally prepared with both types of the lipids i.e. solids as well as liquids. Various advantages of NLCs are discussed below:

1. Enhancement of solubility of the hydrophobic drugs in different dosage forms.
2. Ability to enhance storage stability of different dosage forms.
3. Improvement in permeability and bioavailability of different drugs.
4. Reduction in adverse effects of some drugs.
5. Prolonged biological half-life of drugs.

6. Targeted delivery of the drug to different tissues in the body can be achieved.
7. Sustained release of drugs achieved in the form of coated NLCs

NLCs can be used topically which can reduce the pain as well as discomfort related with intravitreal injections. Because of above mentioned advantages, now a days NLCs are getting more attention.

In one of the studies, researchers repositioned itraconazole (ITR) NLCs to manage DR owing to its potent unutilized anti-angiogenic. High pressure homogenization method was used to prepare ITR-NLCs (Fig. 5a). Tripalimitin (liquid lipid), Capmul MCM (solid lipid) Tween 80 (surfactant) and Transcutol HP (co-surfactant) were used to prepare ITR-NLCs. Further, surface modification of optimized NLCs was done using chitosan to modify the surface charge and to improve the retention and ocular permeability of drug. Anti-angiogenesis study was carried out using HET CAM (Hen's Egg Test – Chorioallantoic Membrane) model, which indicated excellent anti-angiogenic potential of ITR NLCs (Fig.5 b). These NLCs also exhibited anti-neovascularization effect on the rat cornea(Fig. 5 c) [141].

In another study, palmitoylethanolamide (PEA) was found to show the beneficial effects in several retinal diseases such as DR, glaucoma, etc. PEA attenuated the degree of retinal inflammation while preserving the blood–retinal barrier in diabetic rats [142].

Platania et al (2019) developed Myriocin (Myr) loaded NLCs for the treatment of retinitis pigmentosa (RP). The formulation was prepared using melt emulsification and ultrasonication technique using tween-80 as surfactant and Gelucire 44/14 (10% w/v) and Mygliol 812 (5% w/v) as lipids. The Ocular distribution of the Myr-NLC formulation in rabbits and C57BL6J mice was carried out and performance was compared with Myr-aqueous suspension and Myr loaded SLNs. NLC1 formulation provided significantly ($p<0001$) higher myriocin retinal availability, after topical ocular administration, compared to myriocin suspension or Myr-SLN formulation [143].

4.8 Nanogel

Nanogels are nano-sized hydrogels made up of hydrophilic polymers loaded with both type of drugs- hydrophilic and hydrophobic [144,145]. Nanogels can cross the ocular barriers and deliver the drug to posterior segment of the eye especially retina. Nasr et al demonstrated beneficial effect of loteprednol etabonate nanogels eye

drops in DR [146]. Nanogel of methylcellulose hydrophobized with N-tertbutyl acrylamide have also been evaluated for management of ocular diseases [147].

4.9 Cyclodextrin based nanosystems

Cyclodextrin (CD) are made up of cyclic oligosaccharides comprises of six, seven or eight D-glucopyranose subunits. These units have hydrophobic and hydrophilic matrix in the outer part [148]. CD based nano systems can deliver the drug into posterior segment of the eye. These have been explored successfully ocular delivery of drugs for treating ocular diseases [149,150]. Studies on dexamethasone loaded CD micro particles eye drops to delivered the drug into posterior segment of eye for treatment of ocular diseases [151]. In another study, loteprednol etabonate and hydroxylpropyl-β-CD and β-CD were prepared in different forms such as ocuserts, eye drops and gels. These were evaluated for anti-inflammatory activity in conjunctivitis and found effective in reducing inflammatory marker in rabbit's eyes [150]. It is also important to mention that cyclodextrin derivatives-based therapy is currently a promising approach for treating several ocular diseases [152].

4.10 Quantum dots

Quantum dots (QDs) are semiconductor nanocrystals with size between 2-6 nm [153]. They are made up of heavy metals such as cadmium selenide along with zinc sulphide. They are mainly used as imaging agents for labelling glia, neurons [154] and endothelial cells in retinal capillaries [155]. In one of the studies, silicon based QDs were prepared and delivered via intravitreal route for delivering electrical stimulation to retinal cells in model of retinal photoreceptor as neuroprotective agent. These systems have been used for drug targeting into posterior part of the eye for treatment of ocular diseases [156].

4.10.1 Limitations of QDs

Drug loading, release, biodistribution, toxicity, ocular irritation, and patient compliance [157].

5. Miscellaneous

OCT bound magnetic nanoparticles (MNP-OCT) were prepared and tested for treatment of DR. The MNP-OCT were tested *in vitro*, ex vivo, and *in vivo* experimental

models of the mammalian retina to investigate the possible toxicity of MNPs, possible effects of the binding to MNPs on OCT bioactivity, and the localization of MNP-OCT in the retina after intraocular injection. The results indicated that MNP-OCT were non-toxic on human retinal endothelial cells (HRECs) and in mouse retinal explants. Moreover, the binding with MNPs did not influence OCT's antiangiogenic or antiapoptotic activity [158]. The effects of MNPOCT were observed at concentrations up to 100-fold (in HRECs) or 10-fold (in mouse retinal explants) lower compared to OCT, indicating that OCT bioactivity was enhanced in MNP-OCT. MNP-OCT in mouse retinas *in vivo* after intraocular delivery were initially localized mainly to the outer retina, at the level of the retinal pigment epithelium, while after 5 days they were observed throughout the retinal thickness. These observations demonstrated that MNP-OCT may be used as an OCT intraocular delivery system that may ensure OCT localization to the retina and enhanced OCT bioactivity.
In another study, a novel insulin delivering system was developed by loading into chitosan nanoparticles/poly (lactic-co-glycolic acid)-poly (ethylene glycol)-poly (lactic-co-glycolic acid) hydrogel (ICNPH). Subconjunctival route was selected for the ICNPH formulation for administration in DR induced rats and its efficacy was compared with insulin, blank and sham treated animals. The ICNPH significantly reduced the scotopic b-wave amplitude, alleviated retinal micro- and ultrastructural changes, and reduced retinal cell apoptosis affected in DR rats as that of the animals treated alone with insulin [159].

Matrix metalloproteinase-9 (MMP-9) plays a progressive role in the onset and severity of DR. Interleukin-12 (IL-12) is a cytokine of the chemokine family that could reduce the levels of MMP-9 and VEGF-A and suppresses tumor angiogenesis. IL-12-loaded nanoparticles were prepared for long-term and sustained treatment of DR. Their inhibitory effect was evaluated against VEGF-A and MMP-9 expression in rat's endothelial cells and DR induced mouse retina. The nanoparticles significantly reduced retinal damage in DR mice by improving thickness and reducing neovascularization after treatment [160].

Fenofibrate was successfully encapsulated into poly (lactic- co-glycolic acid) (PLGA) NP (Feno-NP) and used for retinal dysfunctions in ocular diseases like DR and AMD. It reduced retinal vascular leakage, inhibited retinal leukostasis, and downregulated

the overexpression of VEGF and intercellular adhesion molecule 1 (ICAM-1) in 8 weeks after single intravitreal injection of Feno-Np and successfully used for the treatment in the DR and AMD [161].

The hybrid aptamer-modified carbon dots (C-dots) have been used as nanomaterials to treat AMD and DR. C-dots functionalized with the VEGF aptamers were used as effective carriers of the anti-VEGF aptamer across the cornea, yielding therapeutic levels via topical route. The formulation showed no toxicity in both *in vitro* and *in vivo* murine animal model. The formulation inhibited VEGF-stimulated angiogenesis in choroidal blood vessels. The efficacy of formulation was found comparable to two commercially available anti-VEGF drugs, bevacizumab and aflibercept [162].

A core–shell triamcinolone acetonide NP containing hydrophobic polycaprolactone core and hydrophilic Pluronic® F68 shell, were prepared as eye drop and evaluated for its efficacy in a DR induced rat model. Results showed improvement of retinal thickness and vascular health of retinal structural and functional activities as compared to DR controls. It also reduced retinal inflammation by decreasing in NF-κB, ICAM-1 and TNFα expression after 20 days of treatment [163].

The suprachoroidal drug delivery utilizing microneedles (MNs) and micro cannulas appear to be minimally invasive procedure for delivering drugs (e.g. triamcinolone acetonide) into retina and choroid [164]. ~~The controlled and localized ocular drug delivery using polymeric eye patch consisting of an array of detachable and biodegradable MNs has been achieved. These MNs could penetrate into the corneal layers and deliver anti-angiogenic monoclonal antibody (DC101) for the treatment of corneal neovascularization (CNV). The MNs were double layered with DC101 to provide biphasic drug release kinetics to enhance the therapeutic efficacy of the MNs. DC101 MNs eye patch produced approximately 90% reduction in CNV in a CNV disease mouse model as compared to a topical eye drop. The researchers also suggest that the MNs patch is minimally invasive and can be self-applied by the patients on their corneas [165].~~ These non-invasive MNs can greatly aid in increasing the bioavailability of a certain drug in a particular tissue by localizing the drug delivery system [166].

Nanowafers (NWs) are small transparent rectangular membranes or circular discs containing drug loaded into nano reservoirs which can be smeared to the ocular surface using a fingertip. NWs improve the controlled drug release by increasing the

residence and contact time of the drug with the corneal and conjunctival surfaces. NWs enhance the drug bioavailability as well as act as a protective polymer membrane to heal injured and abraded corneal surface commonly found in CNV and dry eye disease. These are designed from biodegradable and biocompatible polymers that can be eliminated over the period of time. Dexamethasone loaded NW (Dex-NW) was fabricated using carboxymethyl cellulose polymer and consisted of an array of nano drug reservoirs filled with dexamethasone [167]. The in-vivo efficacy of Dex-NW was tested in a dry eye disease mouse model. Dex-NW was administered as once a day treatment on alternating days for five-day period of time. After the treatment duration it was observed that Dex-NW was able to restore the corneal barrier function along with a healthy ocular surface which was similar to twice a day treatment of topically applied dexamethasone eye drops. Axitinib-loaded NWs were developed for the treatment of CNV. A murine ocular burn model was used to evaluate the in-vivo efficacy of Axitinib-loaded NWs. The laser scanning confocal imaging and RT-PCR results revealed that once a day Axitinib-loaded NWs was twice as effective as compared to axitinib daily topical eye drops.

Various nano-formulations prepared to treat DR are listed in Table 2. Some of the clinical trials conducted for treating DR [168] are listed in Table 3.

Table 2. Nanocarriers used for the treatment of DR

Novel drug delivery system	Preparation method	Drug	Route of administration	Remarks	References
Nanoliposomes	Thin lipid film hydration	Visudyne® (Novartis Pharmaceuticals, USA)	Topical administration	Treatment of predominantly subfoveal choroidal neovascularization in patients with AMD,	[169]
	Extrusion method	Photrex® (Miravant Medical Technologies, USA)	Topical administration	Treatment of AMD, is in Phase III clinical studies	[169]
Nanoparticles (NPs)	Rotary evaporation method	PEG-PLA chains modified with a cell penetrating peptide (CPP)	Intravenous injection	• Reduces neovascular lesion size • Enhanced drug accumulation	[170]
	Extensive analysis	Magnetic nanoparticles	Intraocular delivery	Enhanced drug bioavailability	[171]
	Emulsification evaporation method	Connexin43 mimetic peptide	Intravitreal injection	• Reduces possible ocular complications • Preventing the loss of the photoreceptor	[172]

Solid Lipid nanocarriers (SLN)	Hot homogenization and ultrasonication method	Triamcinolone acetonide	Topical administration	Drug delivery platform for deeper ocular tissues	[173]
	Cold homogenization method	Myricitrin	Oral	• Antioxidant • Antiapoptotic • Antidiabetic	[174]
	Ultrasonic melt-emulsification method	Ciprofloxacin	Intravenous injection	• Controlled release and a superior antibacterial effect	[175]
Nanostructured lipid carriers (NLC)	High pressure homogenization	Itraconazole	Topical	• Antiangiogenic activity • Improved retention and ocular permeability	[88]
	Cold homogenization	Palmitoyl ethanolamide (PEA)	Topical	• Improved corneal permeability • High ophthalmic tolerability • Prolonged retention capacity • Mucoadhesive and film forming properties	[176]

Table 3: Clinical trials reported for treatment of DR

S.No.	Name of clinical Trial	Outcomes	Year of clinical trail	Status	Reference
1	Systemic interleukin 1β inhibition in proliferative diabetic retinopathy: a prospective open-label study using Canakinumab	Systemic IL-1β inhibition has been shown to stabilize retinal neovascular changes in PDR and reduce macular edema	2016	Completed	[177]
2	Effect of Doxycycline vs placebo on retinal function and diabetic retinopathy progression in mild to moderate non-proliferative diabetic Retinopathy A randomized proof-of-concept clinical trial	Low-dose oral doxycycline improves inner retinal function in DR compared to placebo. Although statistical significance was achieved at multiple time points, it was a small, proof-of-concept trial.	2014	Completed	[178]
3	A randomized study comparing the efficacy of bevacizumab and ranibizumab as pre-treatment for pars plana vitrectomy in proliferative diabetic retinopathy	Intravitreal bevacizumab and ranibizumab are equivalent as surgical adjuvants when used as pre-treatment in patients with PDR	2014	Completed	[179]
4	Optical coherence tomography (OCT) for detection of macular	Intravitreal injection of antiangiogenic drugs has also been used to try to	2011	Completed	[180]

	edema in patients with diabetic retinopathy	improve vision in people with macular edema due to DR			
5	Effects of medical therapies on retinopathy progression in type 2 diabetes	Intensive glycemic control and intensive combination treatment of dyslipidemia, but not intensive blood-pressure control, reduced the rate of progression of DR	2010	Completed	[68]
6	Lower somatostatin expression is an early event in diabetic retinopathy and is associated with retinal neurodegeneration	Neuroprotective, antiangiogenic. Downregulated in retinas of diabetics, associated with retinal neurodegeneration	2007	Completed multi-center phase II-III trial	[181]
7	Barbados eye study group hyperglycemia, blood pressure, and the 9-year incidence of diabetic retinopathy: the Barbados eye studies	High blood pressure control (<150/85 mmHg) achieved a 34% reduction in the rate of progression of DR, independent of glycemic control after 7.5 years. These findings were subsequently supported by several studies showing that blood pressure management significantly reduces the risk of progression of DR	2005	Completed	[182]

5. Expert opinion

It is now well established that DR is a microvascular disease with a potential to cause severe vision loss and blindness as well as a devastating effect on quality of life. The increased evidences from the laboratory and clinical reports suggest that neurodegeneration of the retina and inflammation are implicated in pathogenesis of DR with the involvement of RAAS, AGE, PKC, VEGF and inflammatory factors. Development of agents targeting these pathways may provide new therapeutic treatments for DR. In addition, strict metabolic control, maintenance of target HbA1c levels and treatment of associated risk factors, particularly hypertension can be helpful in management of DR. Over the last few decades, there has been immense improvement in the treatment of patients suffering from DR in the form of nanotechnology-based delivery systems. Despite the advancements, there is predicted increase in the number of patients suffering from DR. Retinal delivery by intravitreal and systemic route have few benefits and more risks. A remarkable clinical benefit has been observed with the advent of VEGF therapy in DR patients. However, most of the patients failed to get significant visual improvement. Another major challenge is the cost involved in the therapy, frequent injections, and painful delivery. It is a challenge for group of society who faced financial hardship to be benefited by anti-VEGF therapy. In last five years, the cases of DR have increased tremendously and unfortunately there is lack of cost-effective therapy available in the market to combat the complications arises from disease directly affecting vision. This warrants an urgent need for the development of novel, cost-effective and non-invasive treatments. The delivery of drugs through nanocarriers such as nanoparticles, liposomes, SLNs, and NLCs offers promising alternative to overcome the associated challenges with current therapies. These nanocarriers provide enhanced drug bioavailability, improved permeation of drug to the targeted areas of the retina, enhancement of residence time, non-invasive delivery, better ocular tolerability etc. Despite these advantages, patient compliance and safety always remain a prime concern. Furthermore, advancements in research in the area of gene therapy, ocular implant, stem cell therapy and laser therapy for the treatment of retinal segment of eye are speculated as a game changer in the management of ocular diseases. These developments offer great benefits in providing the drug in a safer, effective and more complaint way. Generation of stable and scalable nanocarrier is always a challenge for the pharmaceutical industries. Moreover, the availability of these advancements for the clinical

use requires utmost efforts of interdisciplinary research collaboration among health care researchers to maintain the vision of diabetes patients at an affordable cost.

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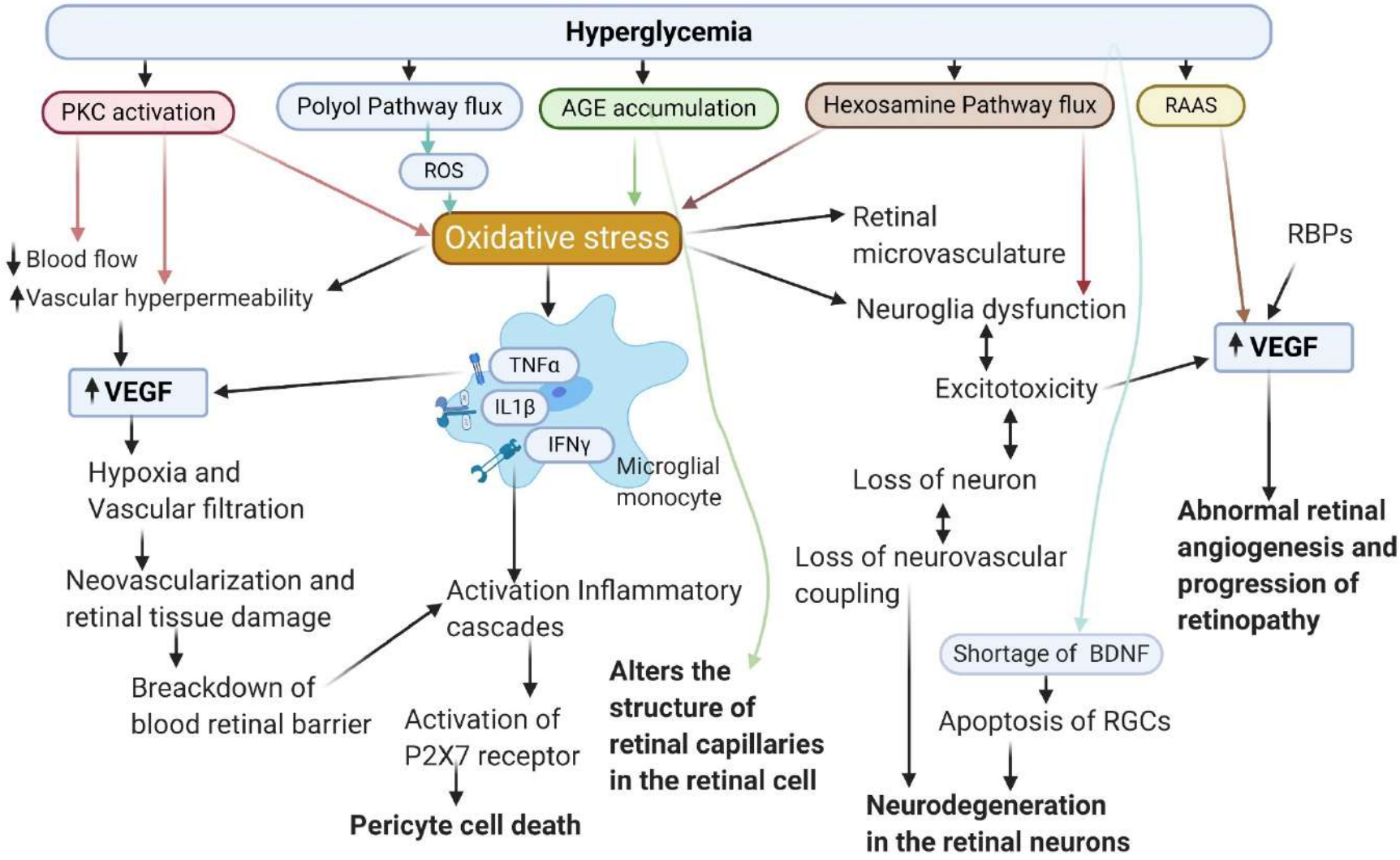

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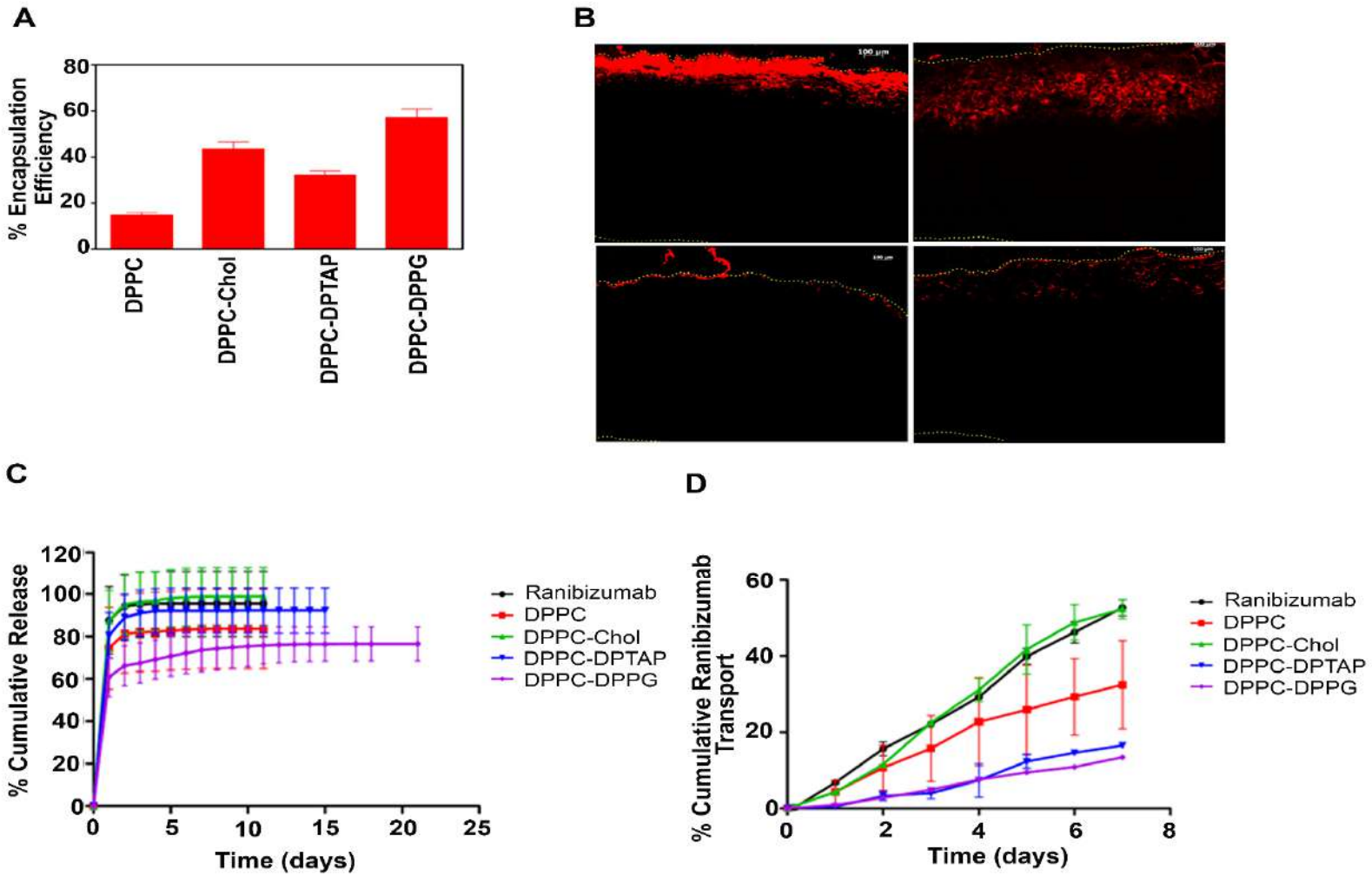

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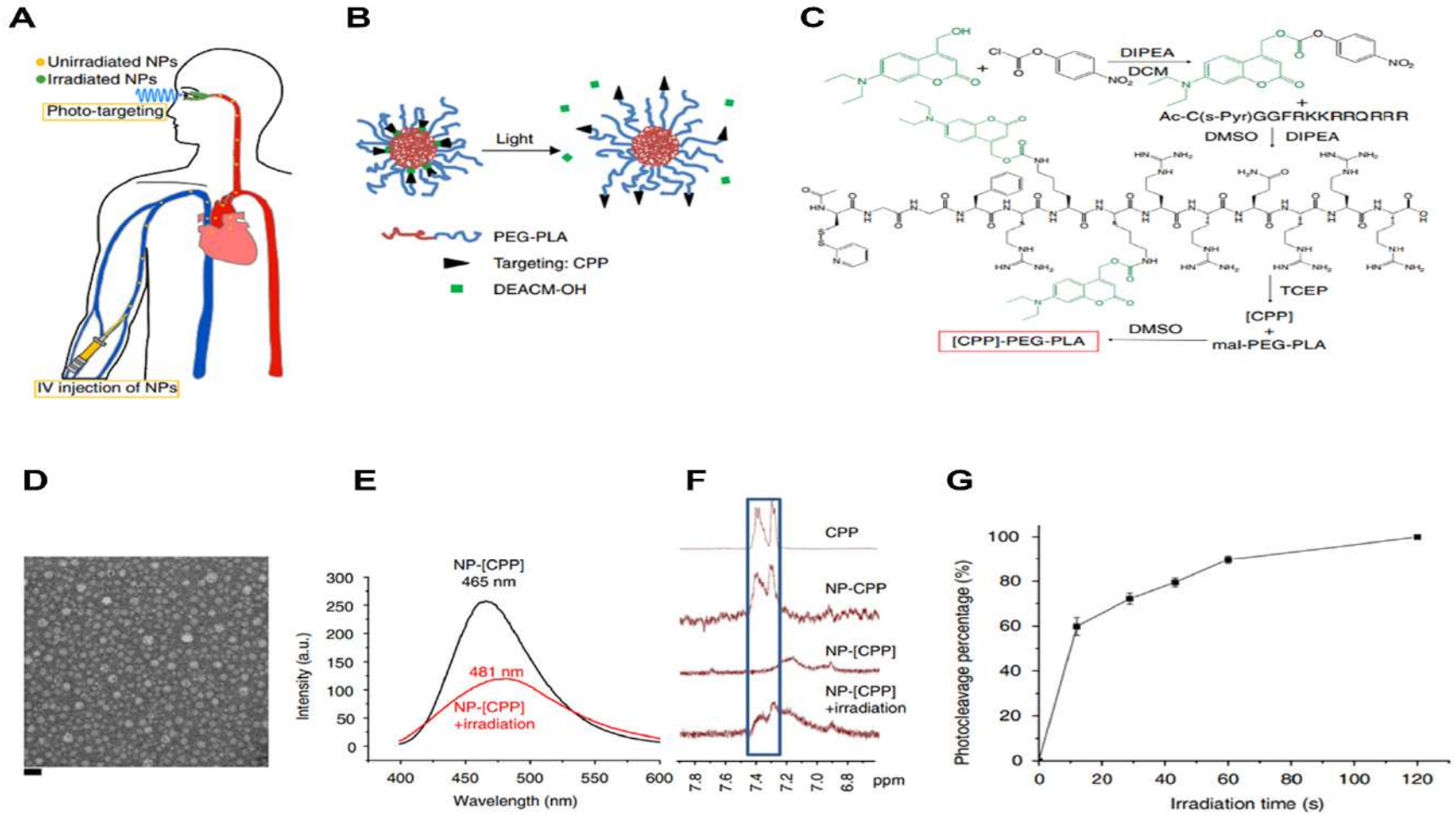

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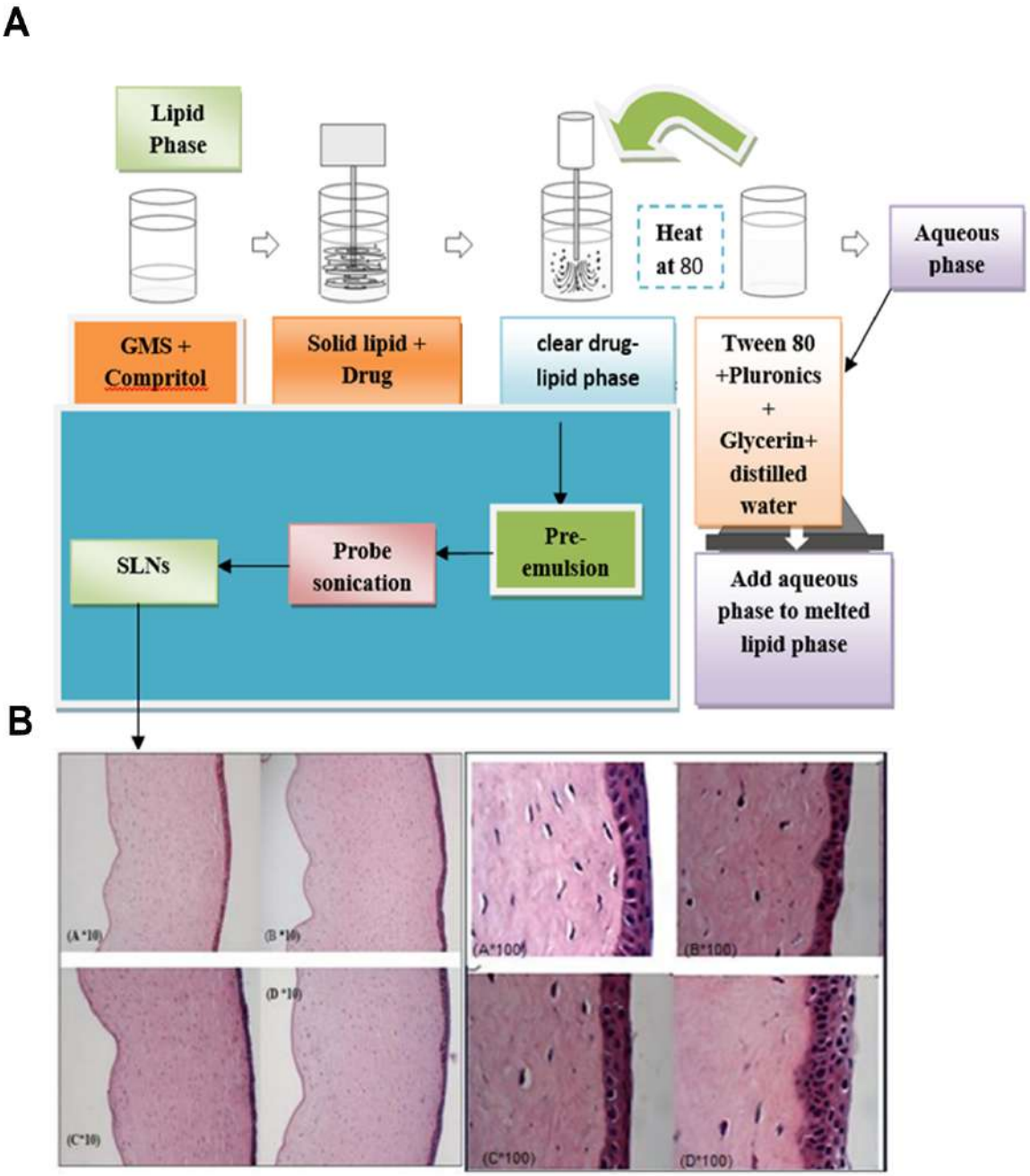

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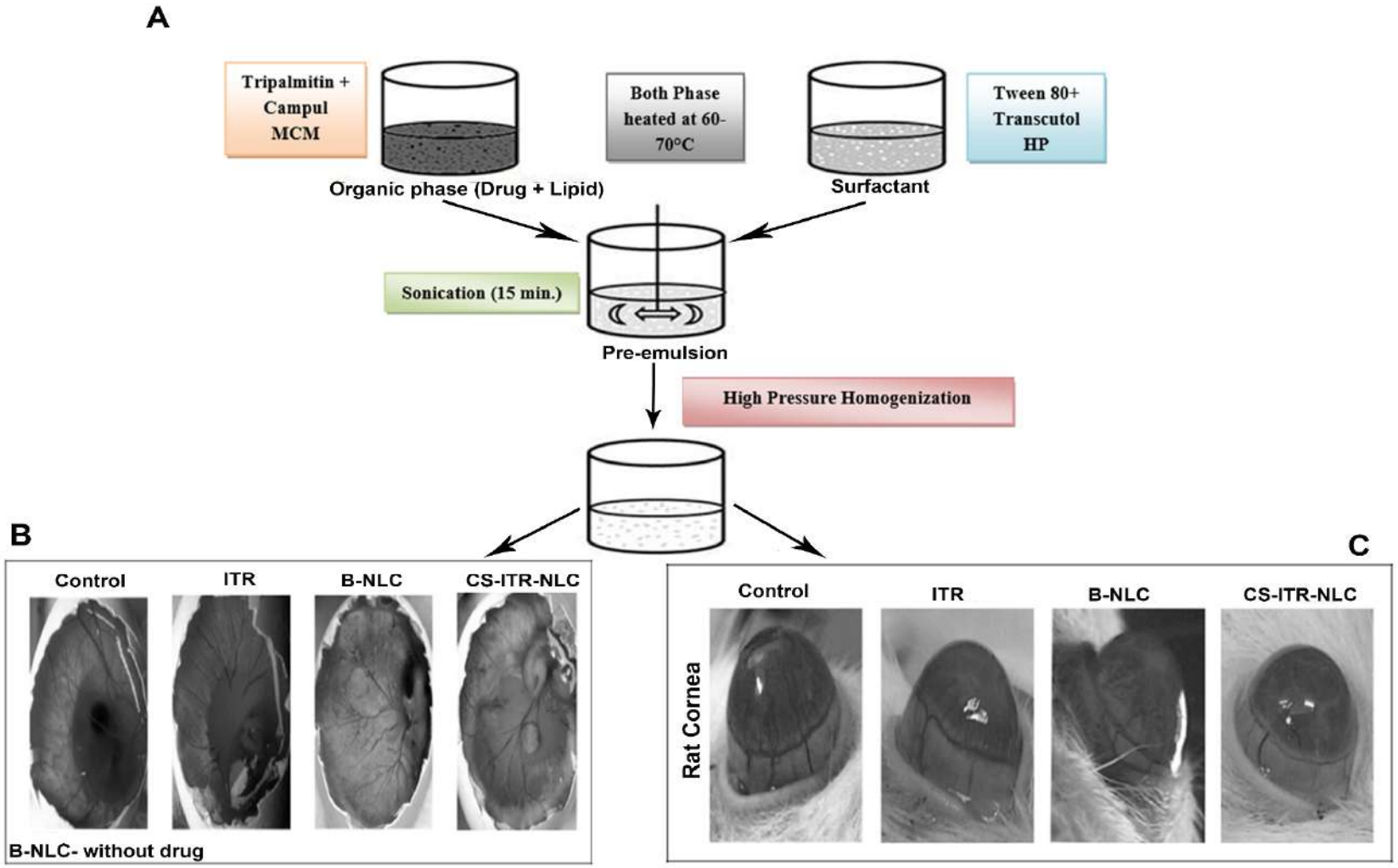

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REVIEW ARTICLE

Current Strategies and Future Perspective for the Effective Treatment of Diabetic Retinopathy

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Abstract: Diabetes retinopathy (DR) is one of the main complications due to diabetes. DR will damage the retinal capillaries and block them, which causes the loss of vision. Different drugs and therapies are used for the treatment and prevention of the DR. The most commonly used treatment is laser technology and combination therapy, along with some drugs. But these drugs possess side effects in the form of cataract, glaucoma, and complete blindness of the eye. The main strategy to overcome In DR, medicines with minimum side effects or maximum therapeutic effects are used. This article emphasizes the current strategy used for the treatment of DR with allopathic as well as herbal drugs.

Keywords: Diabetes, diabetic retinopathy, herbal drugs, laser therapy.

1. INTRODUCTION

1.1. Diabetes Mellitus (DM)

Diabetes mellitus (DM) or Diabetes is a chronic condition in which the human body is not able to produce insulin or it is unable to use insulin which leads to hyperglycemia, *i.e*. the increase in the plasma glucose concentration (as shown in Table **1** below). This hormone is released from the β cells of the islets of langerhens of pancreas which helps in the breakdown of glucose. Hyperglycemia causes other complications like CVS, neuropathy, nephropathy, blindness, diabetes retinopathy, *etc*. [1].

DM is mainly of three types: Type 1, 2, and gestational diabetes. Other types are monogenic and secondary diabetes. The causes and symptoms of the aforementioned types are shown in Table **2**.

1.2. Diabetes Retinopathy (DR)

In India, DR is a major complication of DM which leads to visual impairment. DR damages the retinal capillaries and blocks them, which causes loss of vision. DR is mainly of two types- proliferative and non-proliferative. Further classification of DR is shown in Fig. **1**.

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Proliferative DR

In this type of DR, there is abnormal growth of the blood vessels (also known as neovascularization), which are fragile and can break easily, this causes sudden vision loss.

Non-proliferative DR

This type of DR involves different stages such as-

i. **Mild DR**- Damage of small retinal blood vessels and balloon-like swelling called microaneurysms

ii. **Moderate DR-** Blockage of retinal blood vessels, which causes a decrease in oxygen and nutrient supply to the retina.

iii. **Severe DR**- Retinal ischemia due to the blockage of a large number of retinal blood vessels [1, 2].

2. PATHOPHYSIOLOGY

The pathophysiology of DR is shown in Fig. **2** below.

Diabetes affects millions of people worldwide. It also affects other body parts like kidney, heart, foot, and eyes. Increased level of glucose *i.e*. Hyperglycemia is the main cause of diabetes, which leads to metabolic dysfunction and activation of chronic, low-grade inflammatory signaling which has an important role in DR. Along with medication, some changes in lifestyle helps in the maintenance of BP and sugar level, other activities like daily exercise, taking a balanced

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Table 1. Shows the glucose level in case of diabetes.

Diabetes Conditions	Impaired Glucose Tolerance Conditions	Impaired Fasting Glucose Conditions
During fasting condition plasma glucose level ≥7.0 mmol/L (126 mg/dL)	During fasting condition plasma glucose level <7.0 mmol/L (126 mg/dL)	During fasting condition plasma glucose level is 6.1-6.9 mmol/L (110 to 125 mg/ dL)

Table 2. Classification of diabetes.

Type of Diabetes	Pathophysiology	Causes	Symptoms
Type 1	The autoimmune reaction of the body's immune system on the pancreas, leading to very little or no insulin production	Genetic susceptibility, viral infections, toxins, and dietary factors	Abnormal thirst, dry mouth, sudden weight loss, bed wetting, blurred vision, constant hunger, lack of energy, fatigue, constant urination
Type 2	Insufficient production of insulin and non-responsiveness of the body	Hyperglycaemia, resistant to insulin	Excessive thirst, dry mouth, slow healing wound, frequent and abundant urination, change in vision, foot ulcer, renal failure, or infection.
Gestational	Hyperglycemia during pregnancy	High blood glucose during pregnancy	High blood pressure, fetal macrosomia *i.e.* a large baby which causes difficulty in delivery
Monogenic diabetes	A single genetic mutation in an autosomal dominant	Neonatal DM and maturity-onset diabetes	Excessive thirst and hunger, frequent urination, drowsiness, unconsciousness
Secondary diabetes	Hormonal imbalance	Pancreatitis or Cushing's disease	Increase in thirst, frequent urination, weight loss, tiredness

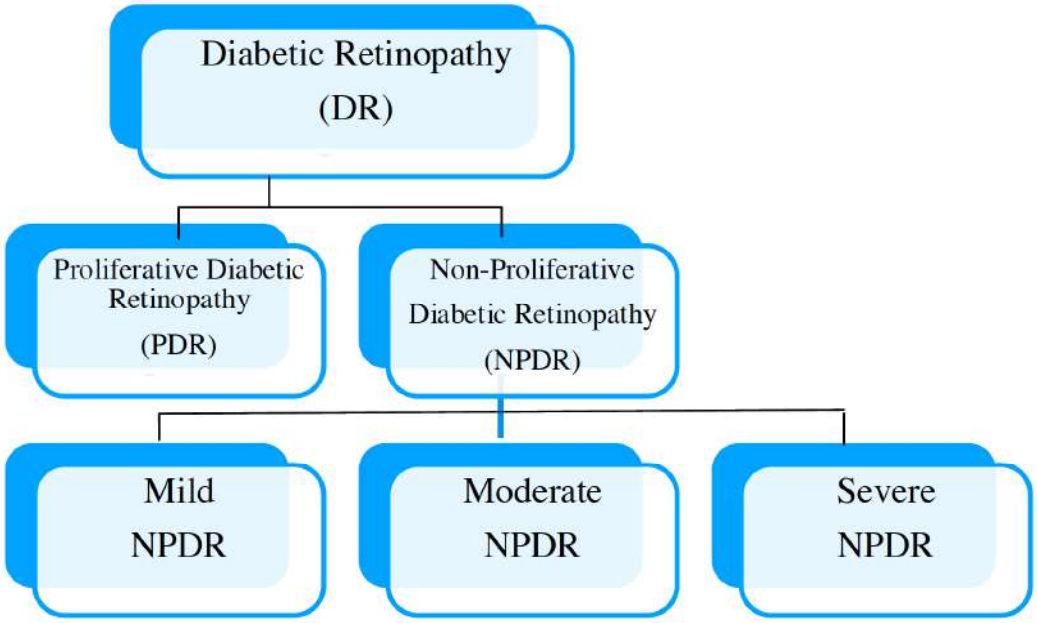

Fig. (1). Classification of diabetic retinopathy.

diet along with fresh fruit and vegetables and reduced amount of fat and sugar are helpful in the management of diabetes. Diabetes can be controlled by reducing the abnormalities related to this which will ultimately cure DR too [3].

Clinical signs of retinopathy include cotton-wool spots, hemorrhages, lipid exudates, microaneurysms, diabetic macular edema (DME), capillary occlusion, neovascularization. Multiple biochemical pathways are involved in the pathogenesis of DR. These include activation of rennin-angiotensin-angiotensinogen system (RAAS) system, the formation of advanced glycalation end products (AGE), and activation of protein kinase C (PKC) isoforms. Furthermore, these events lead to the upregulation of growth hormones and inflammatory factors, such as vascular endothelial growth factor (VEGF). All these factors contribute to physiological, structural and functional alterations in diabetic retina [4].

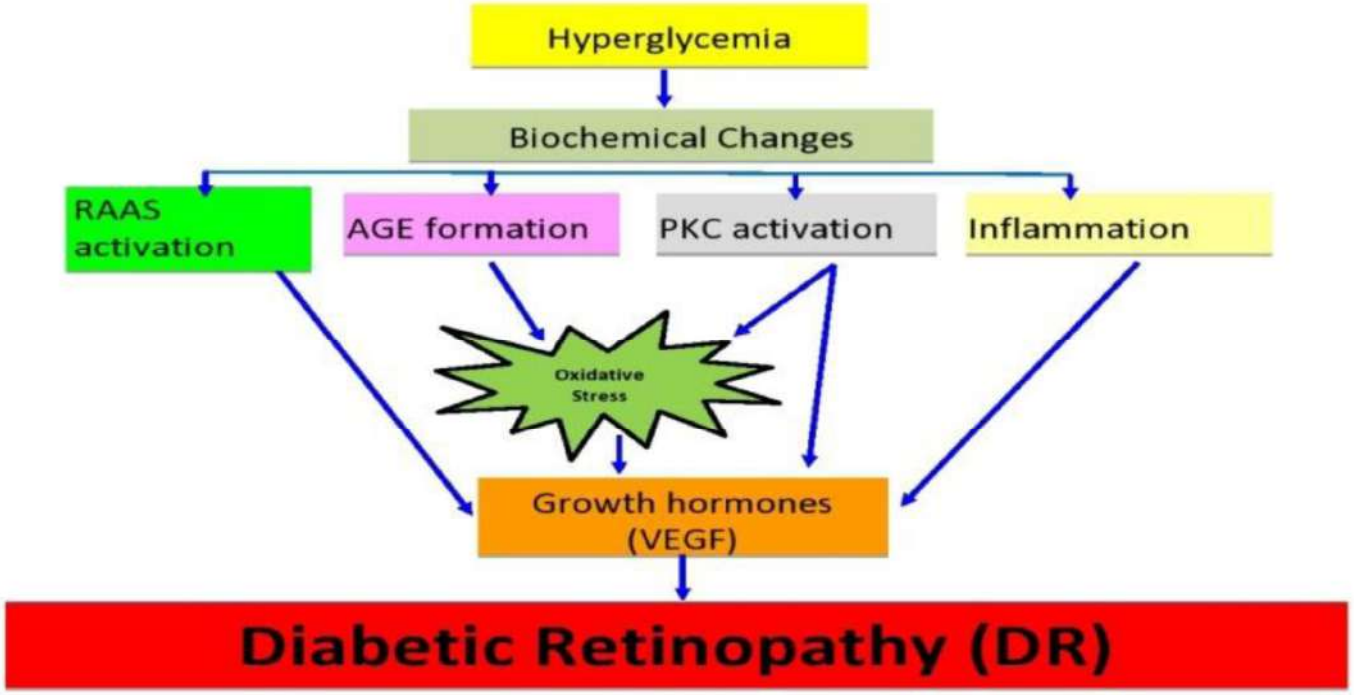

Fig. (2). Diabetic retinopathy pathophysiology.

2.1. Role of Delivery System to Cross Blood-retinal Barrier (BRB)

BRB does not allow the movement of drugs from blood into the posterior part of the eye. The retinal pigment epithelium cells (RPE) and retinal capillary endothelial cells (RCE) are made up of the outer and inner BRBs, respectively. The latter is the peculiar cells present between the choroid and neural retina, which assist the visual system by uptake of retinoids and selective molecular transport between photoreceptors and Choriocapillaries. On the contrary, the tight junctions in these cells impede the intercellular permeation. The outer BRBs, limit the drug entry from the choroid into the retina. Although, a systemic administration of drugs is the ideal route for delivery into the retina but BRB stringently modulates drug penetration into retina from the blood. Therefore, for the transportation of drugs from the choroid into the retina, specific/targeted drug delivery systems or different routes of drug administration, for example, suprachoroidal, systemic, periocular, sub-retinal, or topical are entailed.

Current advancements at nanoscale level have invigorated formulation scientists to overcome BRB. Different nanoparticles, for example, solid lipid nanoparticles (SLNs), polymeric nanoparticles, nanostructured lipid carriers (NLCs), gold nanoparticles, liquid crystals (LC), liposomes and microemulsions. These nanonized systems have several advantages compared to conventional drug delivery systems, such as increased surface area, improved adhesion, depot formation, enhanced biocompatibility and controlled drug release rate. In addition, they reduce the side effects of the drugs and increase patient compliance. Different advancements in the field of nanotechnology to specifically surpass the BRB have been discussed below.

Kim and co-workers investigated gold nanoparticles (GNPs), to cross the BRB, in two different sizes *i.e.* 100 nm and 20 nm and administered them intravenously. They demonstrated that GNPs with a size of 20 nm were uniformly distributed throughout the retina (endothelial cells, neurons, peri-endothelial glial cells) without any cytotoxicity. The authors hypothesized that the size, shape, and chemical composition provided such distribution patterns [5].

Furthermore, the concept of gene delivery has also been explored by the researchers to target retina. The study aimed to transport a non-viral plasmid explicated with gene targeting technology by non-invasive intravenous administration. The pegylated immune liposomes (size 85 nm) encapsulated an expression plasmid, promoter glial fibrillary acidic protein (GFAP) gene, simian virus (SV) 40) and were functionalized with rat 8D3 monoclonal antibody (mAb) in order to target transferrin receptor (TfR) rich structures. The results revealed pervasive exogenous gene expression throughout the retina because of the modulation by cell-specific promoter and elimination of gene expression in peripheral tissues by tissue-specific promoter. Another study reported non-viral retinal gene delivery using arginine–glycine–aspartic acid (RGD) peptide, nano-engineered transferrin, or dual-functionalized poly-(lactide-co-glycolide) nanoparticles administered through intravenous route for effective management of choroidal neovascularization (CNV). In the case of functionalized nanoparticles, intraceptor gene expression in retinal vascular endothelial cells, photoreceptor outer segments, and retinal pigment epithelial cells could be obtained as compared to non-functionalized nanoparticles. A well-established model *i.e.* Laser-induced CNV model was used. The reason for retinal targeting could be attributed to the leaky BRB created by CNV due to laser treatment in rat eye [6]. Campbell and coworkers examined RNAi-mediated

systemic delivery to the retina. The results demonstrated reversible modulation of the inner BRB in mice by repression of the transcript, which encodes a protein component *i.e*. claudin, that forms the paracellular pores and channels for selective ion permeability of the inner retinal vasculature [7].

2.2. Topical Drug Delivery System for Eye Diseases

The topical route (shown in Fig. **3** below) is the most common route for delivery of the drug to the eyes because of its high patient compliance and non-invasive technique. Mostly, the drug is not able to cross the barriers and is unable to reach the posterior eye segment. Nano-drug delivery system aids in better permeability of the drug across the cornea, conjunctiva, and sclera by topical route. The next section describes the different drugs applied topically for efficient delivery into the posterior segment of the eye.

Dexamethasone, a steroid, was formulated with cyclodextrin (water-soluble) microparticles in a form of suspension. The drug was successfully delivered to the posterior part of the eye. In another study, the micellar formulation of dexamethasone and voclosporin were successfully able to achieve therapeutic concentrations and reach the targeted retinal layers [8,9]. Furthermore, the topical application of NSAIDs such as nepafenac decreased VEGF production and prevented choroidal and neovascularization. The study demonstrated that even large molecular weight drugs could not only penetrate the posterior part of the eye but can also be therapeutic [10].

A carbonic anhydrase inhibitor, dorzolamide hydrochloride, was also administered for eye as eye drops and was found to be rapidly distributed in ocular tissues [11].

Furrer *et al*. investigated the difference between systemic and topical administration (eye drops) of a tumor necrosis factor (TNF)-alpha inhibitory single-chain antibody fragment, (scFv) ESBA105 for local drug distribution in a rabbit's eye. The researchers observed a significantly higher concentration of ESBA105 in all the ocular compartments after topical administration. Thus, based on the observed results, the authors proposed ESBA105 as a promising molecule to be delivered through the topical route for the treatment of ophthalmological disorders [12]. Similarly, these two different routes were compared to other routes such as intravenous and intranasal to determine effective dexamethasone concentration. The drug absorption after topical route dominated in the anterior eye segment. A maximum amount of the drug (60%) was released into retina when administered through the topical route.

Betaxolol, an anti-glaucoma drug when applied topically, was found to be localized in the posterior eye segment due to enhanced permeability across cornea and conjunctiva [12].

3. INCIDENCE AND PREVALENCE RATE OF DR

According to a survey done in 2010 in Chipas, United States, it was observed that 38.9% of diabetic people aged around 50 years or older also suffer from DR, out of which, 21.0% suffer from PDR. Diabetes is a major cause of loss of sight and complete blindness in Latin American countries [13].

According to the CDC (Center for Disease Control and Prevention), DR ranks 3rd among all diabetic complications in Mexico. In the region of sub-Saharan Africa, during 2018, total of 2689 patients were studied for DR, out of which 50% were male with an average age of 56 years. Among them,

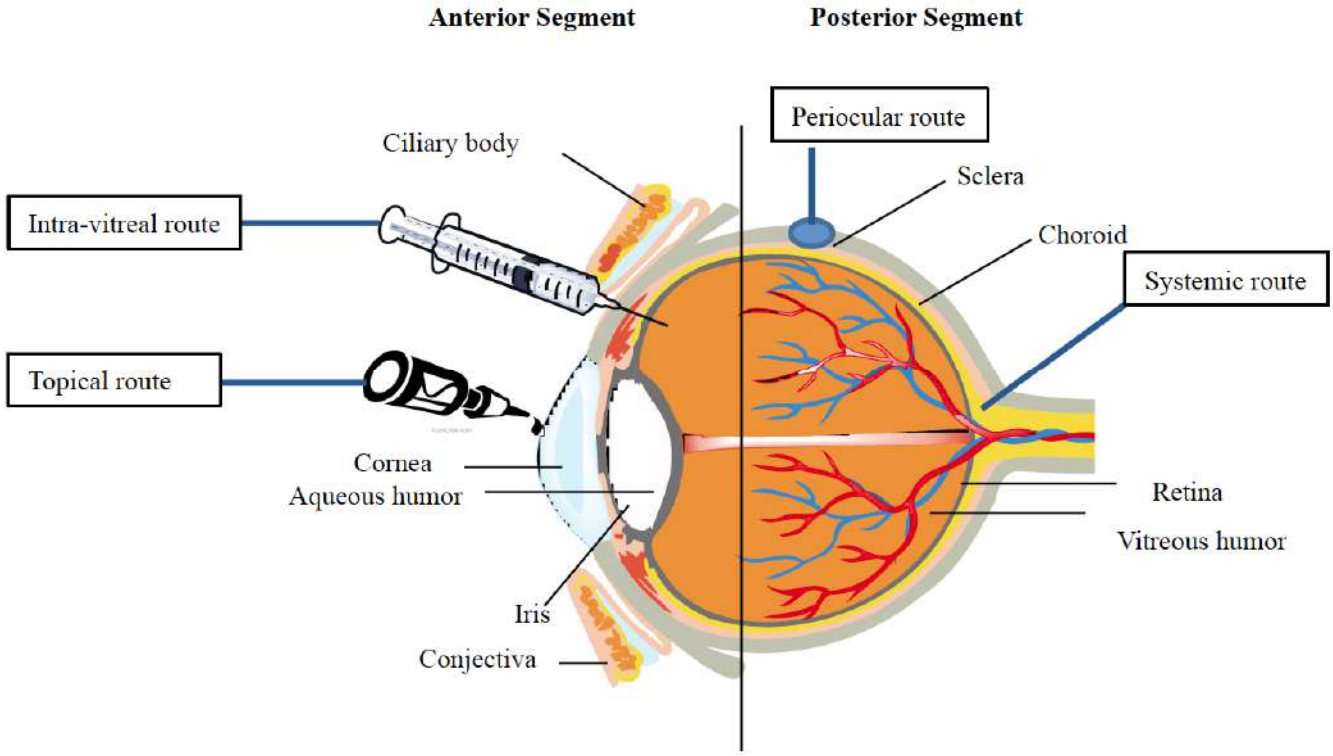

Fig. (3). Different routes and anatomy of eye.

52% were suffering from DR, 36% of patients have DR which is hazardous for eyes, 7% of T1DM and 5% of T2DM had proliferative DR [14].

According to the statement by the ADA, (American Diabetes Association) patients who suffered from PDR/NPDR were treated with laser therapy. It minimizes the risk of loss of vision in people with PDR/NPDR. Anti–VEGF drugs given by intravitreal routes are also used and these are more cost-effective than laser therapy. Also, the optimization of glucose level in the blood, rise or fall in blood pressure, and serum lipid levels with the help of scheduled dilated eye examinations can also decrease the risk of loss of vision in the case of DR [15].

The UK became the 1st country to offer systematic screening of DR for all patients diagnosed with diabetes aged 12 years. All patients diagnosed with diabetes are at risk of DR, however, people suffering from T1DM have a higher probability of DR as compared to T2DM.

In England, the screening of DR was done by the National Diabetic Eye Screening Programme (NDESP), in Wales, it is done by Diabetic Retinopathy Screening Service (DRSSW). In Scotland, screening is run by the Scottish Diabetic Retinopathy Screening (DRS) and in Northern Ireland by the Diabetic Eye Screening Programme (DESP). Pictures of the dilated pupil were taken by non-mydriatic fundus camera to observe the progression of DR [16].

Local therapies used for DR are focal/grid laser therapy, pan-retinal photocoagulation (PRP), and intravitreal anti-VEGF injection. According to the UK Prospective Diabetes Study (UKPDS), metformin helps in reducing the risk of complications related to diabetes when compared with other therapies used for diabetic patients to decrease the blood glucose level [17].

In **Australia**, a DR screening study was carried out for indigenous adults with T2DM in a remote aboriginal community-controlled primary healthcare clinic in Central Australia and certified non-ophthalmic graders in a retinal grading center in Melbourne, Australia. Out of 301 participants, 78.7% had DR with an average age group of 48 (19–86) years and had diabetes up to 9.0 years. The prevalence of DR was 47%. Sight threatening DR has been observed in 78% of detected cases [18].

DR is the main cause of irreversible blindness in adults. The treatment available for DR includes laser therapy, surgery, lifestyle, and suitable medicines used to control BP and sugar level in blood. Different anti-VEGF drugs are also used in the form of intravitreal injections in the eye [19].

In **Canada**, during 2018, higher prevalence rate of DR has been found in the adult population *i.e.* 40.3%, sight-threatening retinopathy is 8.2%. The last prevalence rate of PDR was found to be 23% with T1DM, 14% with T2DM used insulin therapy and 3% used non-insulin anti-hyperglycemic therapies. The treatments for DR include intraocular injection of pharmacological agents, retinal photocoagulation, and vitreoretinal surgery [20].

According to the International Diabetes Federation (IDF) 2017, about 425 million people worldwide were estimated to have diabetes and out of them, about 8.8% of people were in the age group of 20-79 years. Among them, about 79% of the population lives in low and middle-income countries. It is expected that by 2045, about 693 million people between the age group of 18-99 years will be suffering from diabetes [21]. With the increase in disease progression, DM causes complications in the physiological systems of the body irrespective of treatment. DR is one of the common complications among patients with a prolonged history of DM. It damages retinal blood vessels and nerves in the retina which causes vision loss.

Hence, there is an urgent need for the researchers working in the area of DM to understand and rethink the current situation in order to take a step forward for possible treatments for DR.

A study conducted from September 2012 to April 2013 enrolled 105 T2DM in the district of Tamil Nadu, **India**. The prevalence of DR in one eye and both the eyes was 32.53% and 31.58% respectively. The severity of DR was moderate (51.9%) followed by mild (44.4%) and severe (3.7%). DR prevalence was high among people with age >60 years with lesser education level. There was no relationship between DR and its duration, treatment regularity, family history of diabetes, hypertension, visual acuity, and cataract [22].

3.1. All India Ophthalmological Society

All India Ophthalmological Society (AIOS) conducted one study on DR from 14th November to 21st November 2014. Some diabetic patients were analyzed by team members of the society at 194 centers using a structured protocol provided by society. A total of 6218 diabetic patients were observed, out of which 61.2% were males, 88.6% were in the range of 40 and 80 years of age, almost 2/3rd of the patients belong to west and south zones and had diabetes for more than 5 years. DR prevalence in the entire data set was 21.7% and it was high in males [23].

There were over 72 million cases of diabetes in India during 2017. Diabetes is a major cause of complete blindness and one out of three people suffering from diabetes have DR as well. Therefore, there is an urgent need to raise awareness about DR because it is a major health concern for diabetic people. It is the main cause of loss of vision among adults aged 20-65 years. One of the most common and effective forms of treatment is anti-VEGF therapy, which reduces the leakage from the blood vessels and also reduces the growth of abnormal blood vessels in the eye [24].

4. TREATMENT OPTIONS AVAILABLE FOR DR

The treatment or prevention of DR requires synthetic drugs, laser therapy, and combination therapy.

4.1. Role of Synthetic Drugs

These are discussed below in Table **3**:

4.1.1. Anti VEGF Agents

Anti-VEGF drugs like ranibizumab, aflibercept, bevacizumab, and pegaptanib play a very important role in DR treatment. They prevent blindness and improve vision in diabetic patients.

Table 3. Role of synthetic drugs for the treatment of DR.

Drugs (Generic name)	Category	Mechanism	Formulation	Refs.
Avastin (Bevacizumab), Lucentis (Ranibizumab), Eylea (Aflibercept)	Anti VEGF	Reduce macular oedema and proliferation of blood vessels, breakdown of blood retinal barriers (BRB) and increase retinal permeability	Injection into the vitreous cavity of the eyeball	[25]
Pegaptanib		VEGF inhibition from binding and activation of the VEGFR2 receptor	Intravitreal injection	
Dexamethasone	Anti-inflammatory Drugs (Corticosteroids)	Inhibition of leukostasis, mitigation release of local inflammatory factors and enhancement of barrier function of tight junctions	Intravitreal injection	[26, 27]
Fluocinolone acetonide			Implant	
Meloxicam, aspirin, sodium salicylate, and sulfasalazine	Nonsteroidal Anti-Inflammatory Drugs (NSAID)	Inhibit loss of retinal capillaries and inflammation	Topical	[28]
Aminoguanidine	AGE inhibitors	Prevention of AGE accumulation in precapillary arterioles, and decrease vascular occlusion progression	Oral	[29]
Pyridoxamine		Upregulation of laminin protein, and inhibition of AGEs in the retina	Oral	
Ruboxistaurin (RBX)	PKCs inhibitors	Inhibition of PKC 1 and 2 receptors	Oral	[30]
Pazopanib		A selective inhibitor of glycation that leads to the inhibition of VEGF		
Valsartan	RAAS Inhibitors	Angiotensin I (ATI) receptor antagonist	Oral	[31]
Candesartan		Angiotensin receptor blocker		
Lisinopril		Angiotensin-converting enzyme (ACE) inhibition		
Losartan		ACE blocks rennin-angiotensin system		
Miscellaneous drugs	Somatostatin	Antiangiogenic effect	Eye drops	[32]
Fenofibrate	Fibrates	VEGF inhibition, reduction of cytokine levels, PKC activation	Capsule (oral)	[33]
Sorbinil	Aldose Reductase Inhibitors (ARIs)	Decrease the microaneurysms, oxidative stress, basement membrane thickness, neuronal apoptosis, VEGF expression, and gliosis	Oral	[34]
Anti-oxidants	Curcumin, Vitamin C, Vitamin E	Reduction of oxidative stress		

Bevacizumab is a monoclonal antibody injected intravitreously, it prevents the proliferation of blood vessels in the eye by blocking VEGF factors. **Ranibizumab** with **the** same mechanism as that of bevacizumab shows better effect against DR when compared to laser therapy. **Aflibercept** and **Pegaptanib** also have a major role in the prevention of DR as anti-VEGF agents. The use of anti-VEGF drugs can cause serious complications of eyes like impaired wound healing, retinal detachment, endophthalmitis, hypertension, proteinuria, and increased risk of CVS diseases [25].

4.1.2. Anti-inflammatory Drugs

4.1.2.1. Corticosteroids

Dexamethasone is a steroid that can be used in the treatment of DR because it will inhibit leukostasis or enhance the barrier function of tight junctions, and also inhibit the release of local inflammatory factors. But dexamethasone has some side effects such as cataract and an increase in intraocular pressure. **Fluocinolone acetonide** is another steroid which can be used in the form of implants for the treatment of DR [26, 27].

4.1.2.2. NSAIDS

NSAIDS such as salicylate drugs (aspirin, sodium salicylate, meloxicam, and sulfasalazine) can be used for the prevention of DR. These drugs protect the diabetic retina from vascular damage and retinal microangiopathy. **Aspirin** decreases the adhesion of leukocytes to the retina and prevents capillary apoptosis. This work has been reported in streptomycin induced diabetic rat model. Aspirin also has an anti-inflammatory effect which helps in the prevention of the DR. **Nepafenac** act as COX-1 and COX-2 inhibitors and inhibit diabetes-related abnormalities with no side effects on neuron degeneration. **Meloxicam** is also COX-2 inhibitors which inhibit retinal capillary loss in case of diabetic rats [28].

4.1.3. AGE Inhibitors

In diabetes, the rate of formation of AGE increases which leads to DR. **Aminoguanidine** act as (AGE) inhibitors by preventing the accumulation of AGE in precapillary arterioles which decreases the progression of DR. It stops abnormal endothelial cell proliferation and this has been reported in diabetic dogs [28]. P**yridoxamine** is another AGE inhibitor which facilitates the upregulation of laminin protein. Its mechanism has been reported in diabetic rats in which it decreases AGE level in the diabetic rat retina [29].

4.1.4. PKCs Inhibitors

PKC regulates angiogenesis, blood flow, and cell permeability to the eye. PKC activity increases because of oxidative stress on vascular endothelial cells which can be inhibited by PKC inhibitors. **Pazopanib** is a selective inhibitor of glycation that leads to the inhibition of VEGF. **Ruboxistaurin** (RBX) inhibits the PKC 1 and 2 receptor activity and helps in the prevention of DR. It is a well-tolerated drug and also helps in delaying the time of vision loss [30].

4.1.5. RAAS Inhibitors

RAAS helps in maintaining BP and fluid balance in the body. The main enzymes are angiotensin-converting enzyme (ACE), rennin, and type I and type II angiotensin enzyme which show higher levels of prorenin, renin, and Angiotensin-II (AT-II) in the vitreous humor of patients who suffer from DR. RAAS inhibitors can also decrease the level of retinal VEGF in t patients who suffer from DR.

The effect of valsartan was observed in the diabetic rats in which it acts as an AT-II receptor antagonist, which stops the increase in VEGF levels in the retina. **Candesartan** is an AT-I blocker and its effect was seen in diabetic mice retina. Other inhibitors of AT-I (*e.g*., **losartan and candesartan cilexetil**) are undergoing clinical trials for the treatment or prevention of DR. **Lisinopril** also helps in reducing the progression of DR in diabetic people [31].

4.1.6. Miscellaneous Drugs

4.1.6.1. Somatostatin

It is one of the first reported drugs which can be used topically as eye drops for the treatment of DR. It can cross the BRB and reach the posterior region of the eye and can show its pharmacological effect by preventing inflammation and vascular leakage [32].

4.1.6.2. Fibrates

These are lipid-lowering drugs used for the treatment of dyslipidemia. **Fenofibrate** is used for reducing the total cholesterol, low-density lipoproteins (LDL), glycerides, and increase in high density lipoproteins (HDL) levels are due to activation of alpha receptor [33].

4.1.6.3. Aldose Reductase Inhibitors (ARIs)

In diabetes, the amount of glucose increases and the metabolic pathway is activated to produce sorbitol with the help of aldose reductase enzyme which will lead to an increase in oxidative stress. **Sorbinil,** the 1st ARI which is used for clinical trials showed little effect in control and prevented the development or progression of DR [34].

4.1.6.4. Antioxidants

An increase in oxidative stress due to a high blood glucose level leads to other metabolic abnormalities. Some antioxidants such as curcumin, vitamin C, vitamin E, can be used to prevent the oxidative stress for the management of DR [34].

4.2. Role of Herbal Drugs in DR

Some herbal drugs are used in the treatment of DR, these possess fewer side effects as compared to synthetic drugs. Few examples are discussed in this article along with their mechanism as shown in Table **4** below:-

4.2.1. Turmeric

Zhang *et al*; in 2013, explained the mechanism of curcumin in diabetes. Turmeric, a herbal drug, used in the treatment of diabetes in which curcumin is present as a main chemical constitutent which reduces blood glucose level and other diabetes-related disorders such as diabetes nephropathy, diabetes neuropathy, and diabetes retinopathy [35].

Yousef *et al*; in 2013, proposed that in diabetic retinopathy, there is loss of vision because of an increase in the blood glucose level that affects the retina of the eye. Nowadays, laser is mostly used for the treatment of DR, but it has side effects. Therefore, some natural drugs must be explored for the treatment and prevention of DR. Turmeric is one of the safest drugs which can be used in the treatment of DR because of its antioxidant, anti-VEGF or anti-inflammatory activity [36].

Kowluru and Kanwar in 200, examined the curcumin effect on oxidative stress and inflammation and on the retina of diabetic rats. Diabetes was induced by streptozotocin and 0.05% curcumin was given in diet. After the induction of diabetes, the rats were killed and retina was isolated and observed for inflammatory markers and oxidative stress. The results demonstrated that the intracellular antioxidants levels were decreased from 30 to 35% [37].

Deshpandey *et al* in 2017 studied the effect of curcuminoids present in curcumin powder in diabetic rats by using streptozotocin. Curcumin was given to the diabetic rats in their diet in powdered form. Then body weight, food intake and glucose levels of rats were checked weekly. To analyze the effect of curcumin on DR immune histo-chemistry, electro retinogram and immunofluorescence were performed in all groups. It was concluded that soluble curcumin has therapeutic effect on the treatment of DR [38].

4.2.2. Tinospora Cordifolia (TC)

Tinospora cordifolia (TC) is also used in the treatment of DR. Agrawal *et al*; in 2012 studied the effect of TC extract (250 mg/kg) in streptozotocin-induced diabetic rats and biochemical parameters were evaluated. The results demonstrated that VEGF and Protein kinase C level decreased, blood glucose level reduced, TNF-α anti-inflammatory

Table 4. Herbal drugs used in DR.

Drugs (Generic Name)	Mechanism	Major Constituents	Refs.
Turmeric	Antioxidant, anti-inflammatory, anti VEGF	Curcumin	[35-38]
Tinospora cardifolia	Antihyperglycemic, anti-inflammatory, angiogenic, and antioxidant effects	Restores antioxidant enzyme levels, Prevents retinal oxidative stress	[39]
Panax notoginseng	Scavenge hydroxyl and superoxide radicals	Ginsenosides	[40]
Salvia miltiorrhiza	Prevention of angiogenesis and endothelial cell proliferation	Salvianolic acid A, rosmarinic acid	
Lycium barbarum	Upregulation of anti-apoptotic gene expression	Polysaccharides,	
Astragalus membranaceus	Reduction of retinal ganglion cell apoptosis	Polysaccharides, saponins	
Anisodus tanguticus	Prevention of retinal lipid peroxidation	Anisodamine, hyoscyamine, scopolamine	[40, 41]
Puerariae lobata	Decrease the activity of oxidase, VEGF, leakage, and oedema	Puerarin, daidzein, and genistein	[42]
Ginkgo biloba	Reduce the oxidative stress and inflammatory response	Gingolide and flavanoids	

marker decreased, thickened retina, antioxidant enzyme levels were restored, ROS (retinal oxidative stress) and the angiogenic effect was prevented [39].

4.2.3. Panax Notoginseng

Panax notoginseng belongs to family Araliaceae, it has - anti-diabetic effect and can be used for DR as well. The main chemical constituent, saponin, is present in the roots of this plant, *i.e*. ginsenoside Re 14, ginsenoside Rb1, ginsenoside Rg1, ginsenoside Rd, and notoginsenoside, has anti-diabetic effect. These chemical constituents also have anti-inflammatory and antioxidant effects. Its antioxidant activity is helpful in scavenging superoxide radicals and hydroxyl. Furthermore, it prevents the free radical apoptosis of pigmented retina. Its anti-inflammatory activity is helpful in the breakdown of BRB. It can also act as an anti VEGF agent which inhibits the proliferative DR [40].

4.2.4. Salvia Miltiorrhiza

Salvia miltiorrhiza belongs to family Lamiaceae, its dried roots (Danshen) are used for vascular, hematopoietic, and several cardiac disorders. Danshen dripping pills are used in several blood circulation disorders, for example, myocardial infarction, angina, stroke, ischaemic heart disease, and thrombosis. It has anti-inflammatory and antioxidant properties due to the presence of two main chemical constituents-salvianolic acid and rosmaric acid. Salvianolic acid inhibits Ang-II-induced endothelial cell proliferation and angiogenesis, which prevents proliferative diabetic retinopathy. Rosmarinic acid inhibits retinal neovascularization, removes hemostasis in retina, and decreases levels of lipid [41].

4.2.5. Lycium Barbarum

Lycium barbarum belongs to family Solanaceae which has anti-inflammatory, antioxidant, immunoregulatory, hepatoprotective, anti-ageing, neuroprotective, and antiglaucoma action. The main chemical constituents are polysaccharides, cerebroside, carotene, zeaxanthin, betaine, β-sitosterol, p-coumaric, and vitamins. Polysaccharides mainly help in the prevention of oxidative stress-induced retinal endothelial cell apoptosis. Barbarum helps in the prevention of inflammation of retinal tissue, angiogenesis and inhibits the progression of DR [42].

4.2.6. Astragalus Membranaceus

Astragalus membranaceus belongs to family Fabaceae, the roots of this herb have been used for their antioxidant, anti-inflammatory, diuretic, anti-tumour, immune-modulatory, antithrombotic, and antidiabetic properties. The main chemical constituents are polysaccharides, flavonoids, saponins, amino acids, isoflavonoids, volatile oils, sterols, and trace elements. Saponin reduces the retinal ganglion apoptosis and cell proliferation and prevents inflammatory responses. Due to its anti-angiogenic and anti-apoptotic property, extent of retinal damage reduces during diabetes and also the progression of DR [43].

4.2.7. Anisodus Tanguticus

Anisodus tanguticus belongs to family Solanaceae which is mainly used as an anticholinergic agent. It has other uses also such as spasmolytic, antishock remedy, anti-asthmatic, and organophosphate poisoning antidote. It has anti-apoptotic and anti-inflammatory property as shown in Fig. **4** below. The main chemical constituents are tropane alkaloids-anisodine, anisodamine, hyoscyamine, tropine, scopolamine, apoatropine, trichlorophenyl butyryloxytropane and a non-tropane alkaloid cuscohygrine. One study reported that anisodamine increases blood flow and oxygen supply to the retinal tissues and helps in the prevention of retinal lipid peroxidation and ultimately DR I diabetic animal models [44, 45].

4.2.8. Puerariae Lobata

Puerariae lobata belongs to family Fabaceae, it has main chemical constituents like puerarin, daidzein and genistein, which can be isolated from dried root and has vasodilatory, cardioprotective, neuroprotective, hepatoprotective, antioxi-

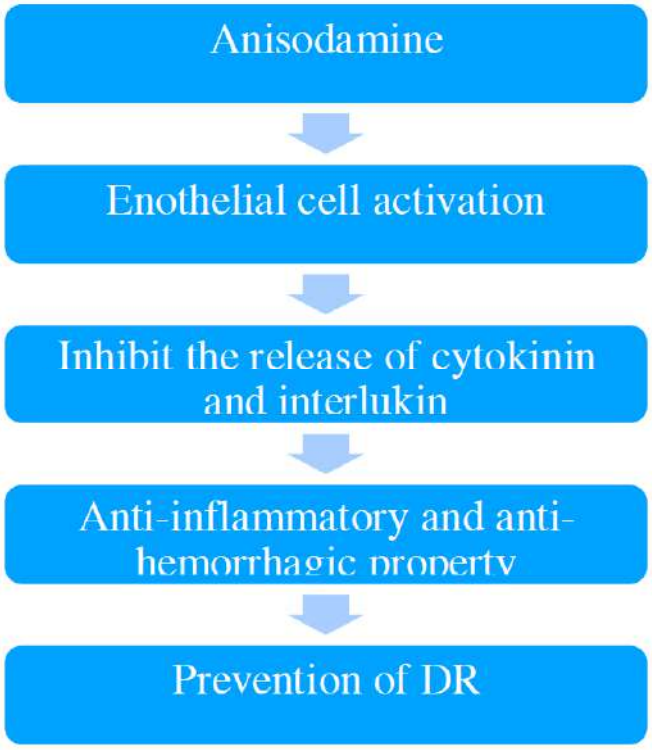

Fig. (4). Prévention of DR by anisodamine.

dant, anti-angiogenic, anti-inflammatory, hypoglycaemic, hypocholesterolemic. It is used for diabetes and for the treatment of its complications. It helps in the protection of retinal cells from apoptosis because of its antioxidant properties. Furthermore, its antiangiogenic property helps in the prevention of DR progression [46].

4.2.9. Ginkgo Biloba

Ginkgo biloba has anti-apoptotic, antioxidant, neuroprotective properties. It also improves blood flow. It can be used for the treatment of diabetes, prevention of DR, asthma, several circulatory disorders and Alzheimer's disease. The main chemical constituents of Ginkgo are biflavones, terpene trilactones, flavonol glycosides and proanthocyanidins. It can also prevent retinal detachment because of its anti-inflammatory effect [47, 48].

4.3. Side Effects of Herbal Drugs

In the treatment of DR, herbal drugs play a very important role. The patients taking herbal drugs along with conventional treatment have better vision as compared to those who rely on conventional therapy only for the treatment of DR. Furthermore, herbal drugs are safe medications without any side effects. Very few side effects of herbal drugs are known such as headache, dizziness, upset stomach, utricaria, *etc*. [49].

4.4. Combination Therapy

Some of the combination therapies (as shown in Fig. **5** below) are also available for the treatment of DR, which shows better results as compared to monotherapy (herbal or synthetic drugs) [50-66].

LIMITATIONS OF EXISTING CONVENTIONAL DRUG THERAPY

There are some difficulties in the treatment of DR which use different conventional systems. They are discussed below:

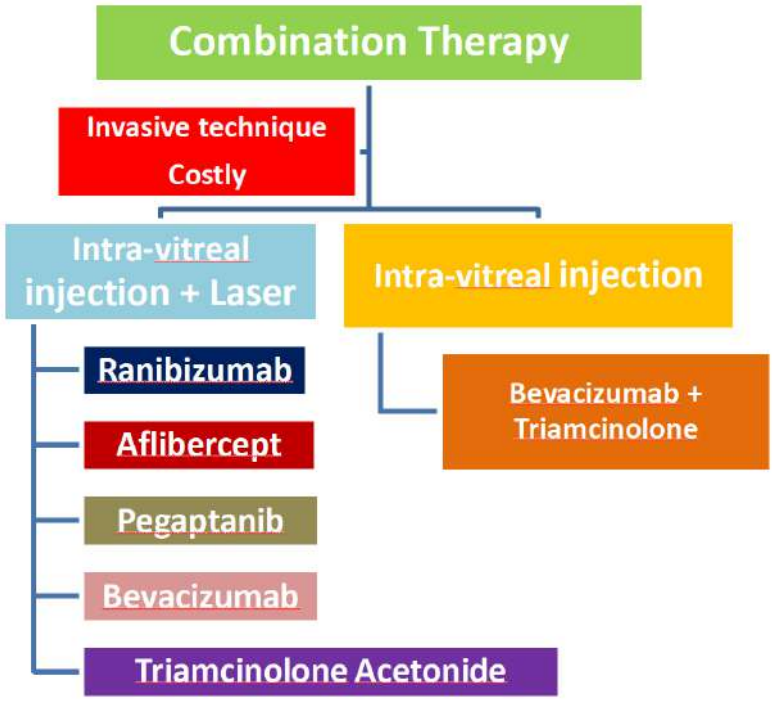

Fig. (5). Combination therapies available for the treatment of diabetic retinopathy.

Eye Drops

While using eye drops, some limitations such as Lachrymal drainage, tear flow, blinking of the eye, *etc*. may reduce the quantity of the drug that reaches the retina.

Intravitreal Injections

Injections are injected directly into the eye, therefore, it is one of the most difficult treatments for DR. It is an invasive, painful and costly technique. Some side effects include reti-

Table 5. Different nanocarriers used for DR treatment.

Nanocarrier	Novel Drug Delivery System	Preparation Method	Drug	Administration	Remarks	Refs.
Liposomes	Liposomes	Dehydration-rehydration method	Bevacizumab	Intravitreal Administration	• Reduction in drug clearance • Sufficient therapeutic concentration of drug for >6 weeks • Enhanced residence time in vitreous.	[68]
	Anionic, cholesterol-fusing liposome	Size extrusion followed by size-exclusion chromatography	Minocycline (ML)	Subconjunctival injection to the retina	• Higher drug concentration achieved in the retina through ML liposomes (delivered intact) as compared to ML alone	[69]
Polymeric nanoparticles	Nanoparticles	Ionotropic-gelation	Betamethasone sodium phosphate	Ocular	• Drug localization achieved in the vitreous humour	[70]
	Tamarind gum based *in situ* gel	Addition of homogenous gum solution to polymeric dispersion	Pilocarpine	Ocular	• Enhanced drug bioavailability	[71]
	Chitosan–sodium alginate nanoparticles	Emulsification evaporation method.	Bevacizumab	Intravitreal injection on retina	• Prolonged duration of action	[72]
Solid Lipid nanocarriers (SLN)	cationic solid lipid nanoparticles	Emulsion evaporation-solidification at low temperature	Tetrandrine	Ocular	• Enhanced bioavailability	[73]
	Lipid nanoparticles	Multiple emulsion technique	Epigallocatechin-gallate	Ocular	• Targeted drug delivery	[74]
Nanostructured lipid carriers	NLC	high pressure homogenization	Triamcinolone acetonide	Ocular	• Improved bioavailability and controlled drug release with no irritancy • Improved adhesion to the ocular surface and interaction with the epithelial membrane • No ocular toxicity and excellent tolerance	[75]
	NLC	Ultrasonication method	Mangiferin	Ocular	• Improved corneal permeability, • High ophthalmic tolerability, and prolonged retention capacity.	[76]
Dendrimers	Poly (amidoamine) (PAMAM) dendrimers	Step by step procedure as described in Dendritech®	pilocarpine nitrate and tropicamide	Ocular	• Encapsulation of poor water-soluble drug into the internal cavities • Increased corneal residence time	[77]
	PAMAM dendrimers	Complexation	Dexamethasone (DEX)	Retina following topical administration	• Anionic DEX–PAMAM complex formulations obtained higher drug concentrations in ocular tissues compared with DEX suspension • Improved bioavailability	[78]
	Cyanine dye (Cy5)-conjugated dendrimer (D-Cy5)	Conjugation between dye and dendrimer	Cyanine dye (Cy5)	Systemic and Intravitreal Delivery	• Both the routes demonstrated similar retinal biodistribution	[79-81]

nal detachment, endophthalmitis, systemic complications like impaired wound healing, hypertension, proteinuria, and increased risk of CVS diseases.

Implants

Few limitations are there related to implants such as Retinal detachment**,** impaired wound healing.

Intravitreal Implants

Dexamethasone has some side effects like cataract and an increase in IOP (Intraocular pressure).

Oral, Intravenous, Subcutaneous or Intra-peritoneal Routes

This route is not efficient for delivery of the drug to the eye because only 1-2% of the drug is able to reach the retina of the eye [67].

NEED FOR ADVANCED DRUG DELIVERY SYSTEM (ADDS)

The challenges of these existing conventional dosage forms suggest that there is an urgent need for ADDS. Some examples (Table **5** above) which are used for the management of DR, aim to reduce the number of injections, and increase therapeutic effect which decreases the side effects and improved patient compliance and they overcome the limitations associated with the conventional dosage forms.

The nanoparticles also act as depots of the drugs, provide a sustained drug release. Different nanocarriers are used for ocular drug delivery such as colloidal carriers; lipid and polymeric nanoparticles as shown in Table **5** above:

FUTURE PERSPECTIVES

The future perspectives of DR involve the development of some novel drug molecules which will target the pathogenesis of DR. For maximum therapeutic effect and minimum side effects, drug should be targeted to the retina in the eye. From eye drops, the drug is not able to reach the retina than other dosage forms available for DR treatment such as intravitreal injections or implants but they have certain limitations. Future therapies for the treatment of DR include monocyte chemo-tactic protein1 (MCP1), Angiotensin 2, matrix metalloproteinase-9 (MMP9), hepatocyte growth factor, kallikrein, and NFkB. The inhibition of hepatocyte growth factor and MMP9 will lead to the prevention of DR or regression of PDR. Retinal neovascularisation was suppressed by NFkB inhibition. Improvement in retinal vascular permeability was associated with Angiotensin 2, Kallikrein activation, and Hepatocyte growth factor. Patients suffering from DR have a high level of MMP9 in vitreous and retina. The regression of DR and PDR can be prevented by using these molecules.

CONCLUSION

DR is a leading cause of blindness and a large population is suffering from this disease. For prevention or control of DR in the diabetic patient's glucose level, lipid level, blood pressure must be decreased. As laser therapy is not very effective, some suitable drugs are used for the treatment of DR. Both herbal and allopathic drugs are used *via* intravitreal route, implants and eye drops Anti-VEGF agents and corticosteroids are mainly used alone or in combination therapy and also with laser therapy.

The best medication for DR treatment should be long-lasting, highly therapeutic, and safe. DR involves inflammation and ischemia. After retinopathy, early and regular treatment must be given to the patient to prevent loss of vision. After treatment, there will be improvement in the vision of DR patient. Therefore, it was concluded that DR can be treated at earlier stages.

CONSENT FOR PUBLICATION

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CONFLICT OF INTEREST

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RESEARCH ARTICLE

Estimation of 5-fluorouracil by high-performance liquid Chromatography Reversed-phase Validated method

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ABSTRACT:
The main intention of the current work is the development of a suitable, simple, precise, reproducible, and accurate high-performance liquid chromatography reversed-phase validated method which can be used for the estimation of 5-Fluorouracil.The estimation was done by high-performance liquid chromatography (HPLC) method. In this suitable stationary phase and optimum composition of the mobile phase was selected which provides good resolution and short run time for the estimation of 5-Fluorouracil (5-FU). Stationary phase used was Nucleodur C18 column (Reverse phase, 250mm × 4.6mm i.d., particle having 5 micron size) and mobile phase consists of combination of Ortho-Phosphoric Acid (OPA) (0.5%) and methanol having ratio 95:5, v/v were used in an isocratic mode of elution. The mobile phase used with a flow rate of 0.8 mL/min and volume of the injection for 5-FU was 20µL. The eluent observed at 266nm for measurement of 5-FU. The validation of this method was carried out with the help of various parameters such as sensitivity, selectivity, system suitability, precision (inter-day and intra-day), accuracy, and linearity according to International Conference on Harmonization guidelines i.e. ICH Q2 (R1). The 5-FU showing retention time at 7.2 min. The responses showing linearity in the concentration range between 2-10µg/mL with correlation coefficient 0.99. The % mean recovery of 5-FU was calculated at three different levels i.e Lower Quality control Concentration (LQC), Middle Quality control Concentration (MQC), High Quality control Concentration (LQC) whose results falls within the range i.e. 95% to 105%, which indicates the accuracy of this method. The % relative standard deviation (RSD) precision (intraday and intermediate) at 3 different levels was < 2% which indicated the precision of this method. LOD i.e. Limit of Detection and LOQ i.e. Limit of Quantification was found to be 0.870277 and 2.637202 respectively for 5-FU.

KEYWORDS: 5-Fluorouracil, RP-HPLC, ICH Guidelines, System suitability, Accuracy, Precision.

INTRODUCTION:
5-Fluorouracil (5-FU) is a pyrimidine analogue (shown in Figure 1 below) and used as anticancer and antimetabolite. 5-FU blocks the thymidylate synthetase enzyme activity which is required for the conversion of deoxyuridylic acid to thymidylic required for synthesis of DNA [1,4]. For estimation of 5-FU a suitable, simple, precise, reproducible, and accurate high-performance liquid chromatography (HPLC) reversed-phase (RP) validated method is required which can be further used for the bioanalytical estimation of 5-FU.

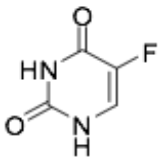

Figure 1: 5-FU Chemical Structure

The importance of analytical method validation in drug development process:
HPLC developed and validated method helps in identification as well as quantification of compounds. As per GMP (Good Manufacturing Practice) requirements different pharmaceutical companies have validation policy to determine the purity of the drug, presence of relative substances, and stability profile of drug [2]. The current validated method also helps in estimation of drug in biological fluids during

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pharmacokinetic, toxicokinetic, and drug distribution study [3]. It also plays an important role in determination of drug loading, and drug dissolution studies [4].

In present research work, different parameters related to the validation of this method such as selectivity, sensitivity, system suitability, precision (inter-day and intra-day), accuracy, and linearity was well explained according to ICH Q2 (R1) guidelines [5]. Validation of method plays a very important role to interpret the data.

The main intention of this research work is development of a suitable, simple, precise, reproducible, and accurate high-performance liquid chromatography reversed-phase validated method which can be used for the estimation of 5-FU [6]. A bioanalytical method could be developed using the same chromatographic conditions for pharmacokinetic study of 5-FU in preclinical and clinical subjects [7].

MATERIAL AND METHODS:

Chemicals and reagents:

5-Fu was procured from LOBA Chemie Pvt. Ltd. Mumbai. Methanol and O-Phosphoric acid (OPA) were of analytical grade and were purchased from Dee-Jay Corporation, Jalandhar. A 0.45µm nylon filter (Pall life Sciences, Mumbai, India) was used. All other chemicals and reagents used were analytical grade unless otherwise indicated.

Instrumentation:

Method Development on RP-HPLC:

HPLC is an instrument which can be used to identify the drug, determine the quantity of drug, and isolation of different components present in mixtures. The HPLC system consists of delivery pump for mobile phase (LC-20 AD; Shimadzu, Japan), a detector (PDA-photodiode array) (SPDM20A; Shimadzu, Japan), a 20µL loop (Rheodyne), and software LC Solution [12]. A Nucleodur C18 column (Reverse phase, 250mm × 4.6 mm i.d., particle having 5 micron size) used as stationary phase and combination of Ortho-Phosphoric Acid (OPA) (0.5%) and methanol having ratio 95:5, v/v used as mobile phase in an isocratic mode of elution [8]. The solvent sample mixture (drug solution) and mobile phase passes through an HPLC column (stationary phase) and then into a detector, where an electronic output is given as a chromatograph signal [9].

Estimation of 5-FU on RP-HPLC:

Different composition of mobile phase solvents system being used, out of which one reliable ratio was selected which provides good resolution and short run time for the estimation of 5-FU. The waste was collected in a vessel outside the machine.

The mobile phase used with flow rate of 0.8mL/min and volume of the injection of 5-FU drug solution was 20 µL. The eluent was observed at 266nm. The retention time of 5-FU was found to be 7.2 min in 20 min run time of HPLC. The peaks of 5-FU in a chromatogram are shown in Figure 2 below. The blank (without drug) was also injected under the same conditions and HPLC was run for 20 min. in which one peak was observed at 4.405 min as shown in Figure 3 below which indicates that drug having a very sharp peak at 7.2 min. only.

The chromatograph signals area i.e. mean peak area was calculated for repeated samples of the same drug solution. For validation of this developed method different parameters were estimated such as selectivity, sensitivity, system suitability, precision (inter-day and intra-day), accuracy, and linearity according to International Conference on Harmonization guidelines i.e. ICH Q2 (R1) [10]..

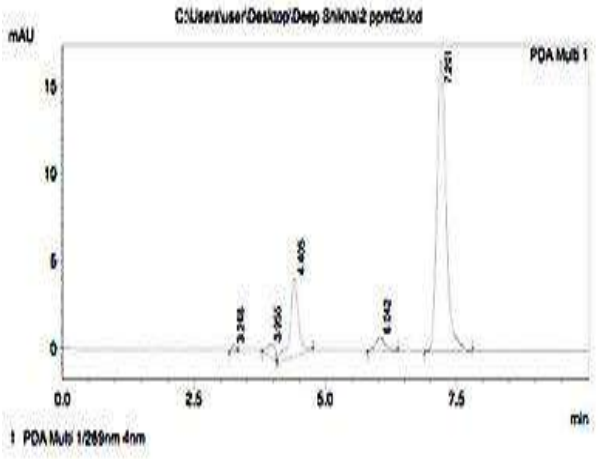

Figure 2: HPLC chromatogram of 5-FU

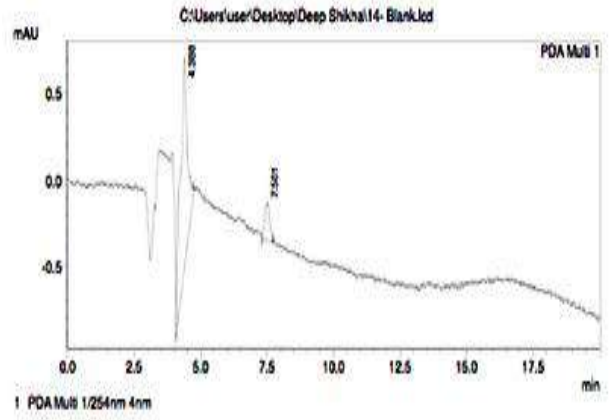

Figure 3: HPLC chromatogram of Blank (Distilled water)

Stock solution preparation:
Stock solution of 5-FU was prepared by accurately weighing drug (10mg) on digital weighing balance and dissolving it in 2mL of distilled water in 10 mL volumetric flask by using vortex. Then make up the volume up to 10mL with distilled water to prepare 1 mg/mL stock solution. Sonicate the stock solution for 10 min. to completely dissolve the drug.

Then, 1 mL of this solution was taken and transferred into another 100mL volumetric flask and diluted up to 100mL with distilled water. Sonicate this solution for 10 minutes to form 10µg/mL concentration. Similarly, other concentrations were prepared such as 2, 4, 6, and 8 µg/mL from the stock solution in different volumetric flasks [11].

Development of calibration curve:
From the above prepared standard drug stock solution 2, 4, 6 and 8 and 10mL was transferred into different flasks (10mL) with the help of pipette and make up the volume up to 10mL with purified water. Sonicate all the samples for 10 minutes. Peak area of all the samples was determined by using HPLC method. Six injections were given for each concentration. The peak of the drug was observed at 266nm and the average peak area was calculated from all six peaks of different concentrations. The calibration curve was plotted between concentrations of 5-FU between 2-10µg/mL drug (x-axis) versus the area of the peak (y-axis). The regression coefficient was found to be 0.99 which shows linearity of the curve [12]. This calibration curve was used further to determine different parameters for validation of the developed method of HPLC.

The proposed work was carried out on a Shimadzu UV-visible spectrophotometer (model UV-1800 series), which possesses a double beam double detector configuration with a1 cm quartz matched cell. All weighing was done on electronic balance (Sansui-vibra DJ-150S-S). A Fast clean ultrasonicate cleaner (India) was used for degassing the mobile phase.

VALIDATION OF METHOD:
Validation was done for the developed method by determining system suitability, linearity, accuracy, precision, LOD, LOQ, precision (inter-day and intra-day), and specificity study as per ICH Q2 (R1) guidelines.

System suitability:
To check system suitability, 6 times injections was given of standard solutions (6µg/mL) of 5-FU was injected to HPLC. The theoretical plates number, peak asymmetry, HETP i.e. height equivalent to theoretical plate and retention time were also measured [13].

Linearity and Range:
The calibration curve was plotted between concentrations of 5-FU between 2-10µg/mL drug (x-axis) versus area of the peak (y-axis) to determine the linearity and range. The regression coefficient was calculated which represents linearity of the curve (4). The range of the analytical procedure was given by the interval between the upper and lower limit of drug concentration. This calibration curve was used further to determine other parameters for HPLC developed method validation [14].

Accuracy:
The accuracy of an analytical method describes the closeness of result between the value which is accepted either as a conventional true value or an accepted reference value and the value found. Accuracy of the experiment was established by using recovery studies from the selected concentration range of calibration curve from 2-10µg/mL, mid concentration of the drug (6 µg/mL) was taken as MQC [100%]. Similarly, LQC [80%] and HQC [120%] of 6µg/mL were also prepared from the stock solution. Suitable aliquots of 4.8, 6.0, and 7.2mL were withdrawn from stock solution and transferred individually into different flasks (10mL) and make up the volume up to 10mL to prepare LQC, MQC and HQC respectively.

Six injections were given repeatedly and the mean area of the observed peaks were calculated for all six injections. For the determination of accuracy of this method mean percentage recovery of the drug was calculated from all these three concentrations. % absolute recovery was calculated by dividing the actual recovery of drug to their theoretical concentration and multiplying them by hundred (Eq.1) [15].

$$\text{Absolute\%recovery} = \frac{\text{Actual concentration recovered}}{\text{Theoretical concentration}} \times 100 \text{--(1)}$$

The data revealed that for all the three levels, the mean % recovery was within the fixed limits of 95-105%. Moreover, the % RSD was also calculated and it was coming in range i.e. < 2%. This indicates accuracy of this method.

Precision:
The precision of an analytical method defined the closeness of results between measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. For determining precision of this method % RSD was calculated for the six observations of LQC, MQC, and HQC solutions at interday (all three dilutions made in three different days), intraday (all three dilutions made within same

day) and interanalyst (all three dilutions made by three different analysts) with same experimental conditions. The % RSD for all the samples was within the limit i.e. RSD < 2%. This proved this method was sufficiently precise [16].

Estimation of LOD and LOQ:

The LOD i.e. Limit of Detection of an analytical method is defined as the lowest concentration of sample or drug which can be detected**.** The LOQ i.e. Limit of Quantification of an analytical method is defined as the lowest concentration of sample or drug which can be determined quantitatively**.** Both were calculated to determine sensitivity of this method by using standard deviation of response (σ) and slope of standard curve (S). Standard deviation of Y intercepts of regression line was used as standard deviation [17]. Eq. 2 and Eq. 3 for LOD and LOQ, respectively as follow:

$$LOD = 3.3\frac{\sigma}{S} \quad (2)$$

$$LOQ = 10\frac{\sigma}{S} \quad (3)$$

APPLICATION OF VALIDATED METHOD:

The developed and validated method will be used for estimation of percentage of drug loaded in Nano Lipid Carriers i.e. NLCs, assay of tablet formulation, percentage of drug released during dissolution with respect to time, and drug diffused through goat cornea. The estimation of drug in biological fluids during pharmacokinetic, toxicokinetic, drug distribution study can be done by using this validated method [18].

RESULTS AND DISCUSSION:

HPLC method development for 5-FU estimation:

Different compositions of mobile phase in varying ratios and flow rate were used. Among them, the compositions having mobile phase combination of OPA (0.5%) and methanol with ratio 95:5, v/v and 0.8 mL flow rate which provide acceptable chromatograms. Hence, it was decided to develop chromatograms with these conditions. The retention time for 5-FU was observed at 7.2 min in the obtained chromatogram with the help of HPLC [19].

System suitability:

Number of theoretical plates, tailing factor, peak asymmetry, HETP, and resolution were measured. All the parameters were found within the limits as shown in Table 1 below.

Table 1: System suitability parameters

Parameters	5-FU
HETP	18.883
Theoretical plate	7943.607
Tailing factor	1.294
Resolution	7.201

Linearity and Range:

The graph was plotted between the concentration of drug and mean peak area to obtain the calibration curve [20]. 5-FU calibration curve showing linearity in the concentration range from 2-10µg/mL having 0.99 regression coefficient as shown in Figure 4 below.

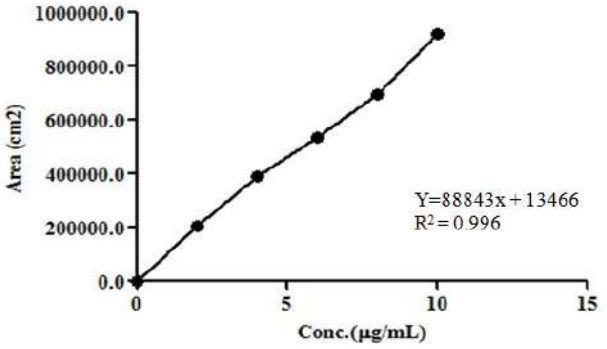

Figure 4: Standard curve of 5-FU

Accuracy

Accuracy was observed by calculating mean % recovery of the drug from HQC, MQC, and LQC solutions containing 6 µg/mL of sample solution. The data revealed that for all the three levels, the mean % recovery was within the fixed limits of 95-105 % (Table 2). Moreover, the % relative standard deviation (RSD) was < 2%. This indicates the accuracy of the developed method.

Table 2: Precision data of the proposed HPLC method from the standard solution of 5-FU

Level	Conc (µg/mL)	Mean peak area (N=6)	% RSD	% recovery	Mean % recovery
LQC	4.8	418793.3	1.16	95.05	96.92
MQC	6.0	520793.8	1.84	95.17	
HQC	7.2	656759.0	1.62	100.56	

Precision:

Precision of this method was observed by calculating the % RSD for the 6 injections of the HQC, MQC, and LQC solutions at inter day, intraday and inter analyst by using same experimental conditions. The calculated % RSD for all the samples showing < 2% RSD (Table 3). This proves that the developed method was sufficiently precise.

Table 3: Precision data of the proposed HPLC method from the standard solution of 5-FU

Parameters	Levels	Conc. (µg/mL)	1	2	3	4	5	6	Mean area (N=6)	Standard Deviation	% RSD	% Recovery
Repeatability (Intraday precision)												
	LQC	4.8	426409	421031	413124	415398	420817	415981	418793.3	4871.952	1.16	95.05
	MQC	6.0	520272	507850	511819	531648	522721	530453	520793.8	9624.855	1.84	95.17
	HQC	7.2	647909	645153	665459	648195	667899	665939	656759.0	10681.070	1.62	100.56
Intermediate Precision (Interday)												
Day 1	LQC	4.8	427504	427571	421298	420149	420813	420945	423046.7	3498.514	0.82	96.04
	MQC	6.0	578362	577859	567830	575905	576280	578247	575747.2	4013.395	0.69	105.00
	HQC	7.2	641328	623472	632932	635493	632796	621622	631273.8	7456.281	1.18	96.50
Day 2	LQC	4.8	427484	423376	420025	421107	420280	420553	422137.5	2633.264	0.62	95.83
	MQC	6.0	529125	521914	526259	526612	526735	526735	526230.0	2352.263	0.44	96.10
	HQC	7.2	658315	652603	653486	640909	647474	663877	652777.3	8042.184	1.23	99.94
Day 3	LQC	4.8	426409	421031	413124	415398	420817	415981	418793.3	4871.952	1.16	95.05
	MQC	6.0	520272	507850	511819	531648	522721	530453	520793.8	9624.855	1.84	95.17
	HQC	7.2	647909	645153	665459	648195	667899	665939	656759.0	10681.07	1.62	100.56
Intermediate Precision (Inter-analyst)												
Analyst 1	LQC	4.8	422626	425254	418589	422869	411314	416989	419606.8	5065.722	1.20	95.20
	MQC	6.0	576697	578655	567866	577069	576541	553699	571754.5	9636.609	1.68	104.70
	HQC	7.2	668853	657830	657326	639130	651552	644430	653186.8	10593.110	1.62	100.00
Analyst 2	LQC	4.8	421879	422209	424847	422832	420616	421308	422281.8	1467.903	0.34	95.86
	MQC	6.0	526072	529951	528316	514351	523912	518729	523555.2	5967.287	1.13	95.69
	HQC	7.2	625902	622961	627159	622389	624441	624331	624530.5	1782.537	0.28	95.50
Analyst 3	LQC	4.8	417612	424204	423769	420214	429046	415422	421711.2	4956.752	1.17	95.73
	MQC	6.0	535344	524297	522601	531344	527763	515741	526181.7	6906.219	1.31	96.18
	HQC	7.2	658743	663460	664909	652526	662644	661976	660709.7	4502.980	0.68	101.18

Estimation of LOD and LOQ:

LOD and LOQ were determined by the slope of standard curve (S) and standard deviation of response (σ) were as follow [20]:

$$LOD = 3.3\frac{23429.69}{88843} = 0.870277\,\text{ng/mL}$$

$$LOQ = 10\frac{23429.69}{88843} = 2.637202\,\text{ng/mL}$$

CONCLUSION:

In this research, a successful RP-HPLC method was developed for the estimation of 5-FU. The developed method was validated for linearity, range, precision, accuracy, system suitability, LOD, and LOQ as per ICH guidelines. The selected method conditions and mobile phase composition provides good resolution and short run time (around 7 min.) for estimation of 5-FU. The results of this work reflected that current method is free from interference of the impurities during the estimation of 5-FU. The low RSD values for all parameters confirmed the validity and reliability of method. All the results manifested that developed method is selective, precise, accurate and linear over the concentration range of 2-10 µg/mL. The method was found to be appropriate for estimation of 5-FU in bulk and pharmaceuticals. In future, a bioanalytical method could be developed using the same chromatographic conditions for pharmacokinetic study of 5-FU in preclinical and clinical subjects.

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CONFLICT OF INTEREST:

The authors declare no conflict of interest.

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CHAPTER

15

c0015

Role of novel drug delivery systems in overcoming the challenges associated with intraocular delivery of drugs: an overview

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OUTLINE

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s0010

1. Introduction

s0015

1.1 Eye anatomy and physiology

p0165 The organ of sense of sight is eye which is found in orbital cavity abounding with optic nerves. Eye has spherical shape and its diameter is about 2.5 cm (1 inch). It is protected with the help of fats along with bony walls of orbit [1]. It is often compared with camera and out of five sensory organs it is utilized the most. About 75% of the information, we receive around us is through visual information [2]. Around one-sixth of the anterior part of eye is exposed and extrinsic muscles of eye are composed of superior rectus, lateral rectus, inferior rectus, medial rectus, inferior oblique, and superior oblique [3]. The eye has different parts that play an important role such as eyebrows, eyelids, conjunctiva, lachrymal apparatus, and eyelids margins (shown in Fig. 15.1) [4]. There are two eyebrows present over the eyes that act as shields and provide protection to the eyes against dust, foreign particles, sweat, water, etc. Eyes can be closed with the help of eyelids which also has protective action against dust, water, and any foreign particles by blinking. Eyelids are movable portions that have eyelashes on them. It is composed of muscles, connective tissues, and conjunctiva. Conjuctiva is a transparent membrane which is composed of epithelium and is present in the front of retina. Lachrymal apparatus consist of lachrymal sac, nasolachrymal duct, lachrymal canaliculi, and lachyrmal gland, which provides protection to the eye by secreting tears and oily substances whenever there is any irritation due to foreign particles or any chemical. Eyelid margins consisting of sebaceous glands which secrete oily substances for lubrication so that dryness of the eye can be prevented [5].

s0020

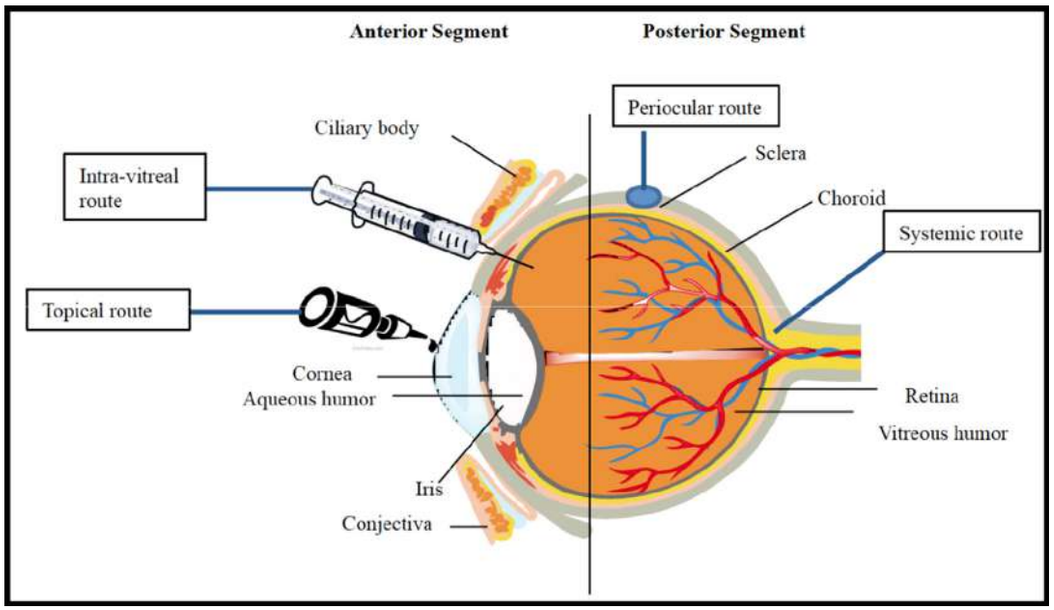
1.2 Diseases associated with eyes

p0170 There are various common diseases associated with eye like diabetic retinopathy (DR), age-related macular degeneration (AMD), glaucoma, dry eye, cataract, iritis, etc. These diseases result in vision impairment or even permanent blindness.

s0025

1.2.1 Diabetic retinopathy

p0175 Among these diseases, DR is posterior eye related disease in which there is damage to the retinal blood vessels due to increase in glucose level in body [6]. The prevalence rate of



f0010 FIGURE 15.1 Different parts and anatomy of eye.

retinopathy, a familiar microvascular complication of diabetes mellitus is very high. It is categorized into nonproliferative diabetic retinopathy (NPDR) as well as proliferative diabetic retinopathy (PDR), based upon the growth and development of new blood vessels [7].

s0030 ### 1.2.2 Age-related macular degeneration

p0180 During AMD various major abnormalities occur in four tissues, i.e., retinal pigment epithelium, photoreceptors, Bruch's membrane and choriocapillaris. AMD is basically classified into two types: dry AMD and wet AMD [8].

s0035 ### 1.2.3 Iritis

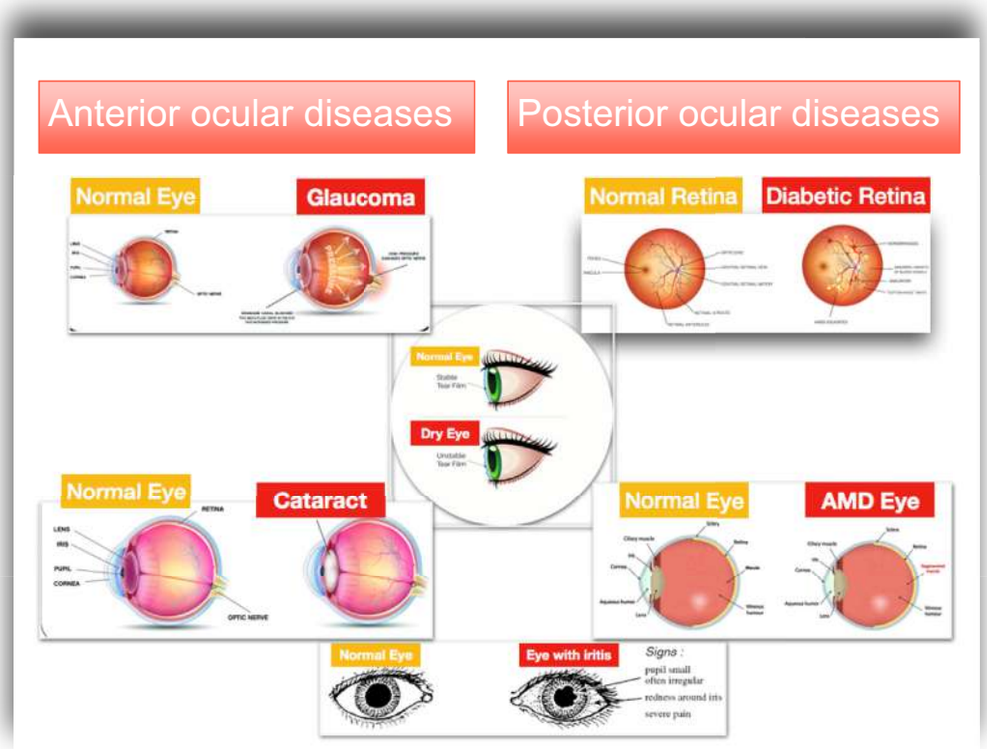
p0185 Iritis is also known as acute anterior uveitis and there is inflammation of either anterior or posterior portion of iris. Its etiology is unknown for most of the cases (idiopathic, around 80%) and rest 20% cases are due to the forced trauma to eye. Drugs like corticosteroids and immunosuppressive agents are administered to treat iritis [9].

s0040 ### 1.2.4 Glaucoma

p0190 It is another eye related disease which causes permanent blindness due to increased intraocular pressure (IOP) which causes damage to optic nerves [10].

s0045 ### 1.2.5 Cataract

p0195 A cataract is an ocular disease in which dense, cloudy area is formed in the lens of the eye. It is caused due to proteins which form clumps thus preventing the lens from sending clear images to the retina [7,9,11].



f0015 FIGURE 15.2 Anterior and posterior ocular diseases.

s0050 ***1.2.6 Dry eye***

p0200 It is an eye disease in which there are abnormal ocular/eye sensations which can be dryness, allergy, discomfort and pain. Also there is decrease in production of tear and increase in tear evaporation [12].

p0205 Brief description of various diseases are detailed in Fig. 15.2 [5,7].

s0055 2. Conventional therapies for ocular diseases

p0210 Ophthalmic preparations are mostly given in semisolid form or liquid form meant for administration of drugs into eye cavity present in between eye lids and eye balls. Some examples of conventional ocular drug delivery system are eye drops, eye solutions, eye ointments, eye suspensions and contact lens solution.

s0060 2.1 Eye drops

p0215 Ophthalmic drops are mostly sterile, aqueous or oily preparations or suspensions or emulsions of single drug or combination of drugs. These are isotonic and sterile formulations with respect to eye and pH value should be in between 4-8 which is tolerable by eye otherwise there may be discomfort, irritation and decrease in bioavailability due to increased tearing [13].

s0065 2.2 Eye solutions

p0220 Eye solutions are also known as eye lotions or ophthalmic solutions. These are sterile, aqueous preparation utilized for cleaning and rinsing of eyeballs. The most common active ingredients include polyethylene glycol, polyvinyl alcohol, propylene glycol, carboxymethylcellulose, povidone, glycerine, and mineral oil [14].

s0070 2.3 Eye ointments

p0225 Eye ointments are semisolid preparations consisting of solid or semisolid hydrocarbon base. After applying it converts into droplet form, having more bioavailability but main disadvantage is that it causes blurring of vision [15].

s0075 2.4 Eye suspensions

p0230 Eye suspension contains solid drug dispersed in vehicle and are homogenous in nature. But main concern is that particle size should be kept in consideration while preparing suspension as large size of particle can cause irritation to eye [16].

s0080 2.5 Contact lens solutions

p0235 Contact lens solutions are also sterile preparations and are isotonic in nature intended for used as wetting solution and sometimes as storage solutions [10,17].

p0240 There are several ocular treatment options in form of synthetic as well as herbal drugs which are discussed in Tables 15.1 and 15.2 respectively along with some marketed formulations related to ocular diseases for treatment of eye related problems.

s0085 2.6 Role of herbal drugs in ocular diseases

p0245 Some herbal drugs are used in the management of ocular drugs, these possess fewer side effects as compared to synthetic drugs. Few examples are discussed in Table 15.2 below:

s0090 3. Challenges associated with conventional therapy related to delivery of drugs at posterior segment of eye

p0250 Conventional systems which includes ophthalmic drops, eye suspensions, eye emulsions, and eye ointments are not considered most advantageous for treatment of ocular diseases as

t0010 **TABLE 15.1** Marketed formulations for ophthalmic drug delivery systems.

S.No	Ocular diseas	Drug	Dosage form	Brand name	References
1	Glucoma	Timolol maleate	Solution	Poentimol	[18]
		Brinzolamide	Suspension	Azopt	
		Dorzolamide and timolol	Eye drops	Cosopt	
		Travoprost		Travatan	
2	Dry eye	Timolol maleate	In situ gel	Timolol GFS	[19]
		Carbomer	Bioadhesive gel	Geltears	
		Cyclosporine	Emulsion	Restasis	
		Hydroxy propyl methyl cellulose	Eye drops	Refresh tears	
		Carboxymethyl cellulose		C-nac	
3	Diabetic retinopathy	Bevacizumab	Intravitreal injections	Avastin	[20]
		Ranibizumab		Lucentis	
		Aflibercept		Eylea	
		Pegaptanib sodium		Macugen	
		Flucinolone acetonide	Intravitreal implants	Iluvien, Retisert	[21]
		Ganciclovir		Durasert, Vitrasert	
		Dexamethasone (corticosteroid)		Ozurdex	
		Triamcinolone acetonide (TA)		I-Vation	
		Dexamethasone (corticosteroid)	Emulsion	Cortiject	
4	Cataract	Potassium iodide	Eye drops	Catlon	[22]
		N-acetylcarnosine		Can-C	
		Pirenoxine		Clarvisan	
5	Iritis	Dexamethasone, PLGA, and HPMC	Implant	Surodex	[23]
		Dexamethasone (corticosteroid)		Ozurdex (posurdex)	
			Intravitreal emulsion	Cortiject	
		Triamcinolone acetonide (TA)	Intravitreal injection	I-Vation	
6	AMD	Bevacizumab	Intravitreal injection	Eylea	[20]
		Ranibizumab		Avastin	
		Aflibercept		Lucentis	

TABLE 15.1 Marketed formulations for ophthalmic drug delivery systems.—cont'd

S.No	Ocular diseas	Drug	Dosage form	Brand name	References
		Perfluorohexyloctane	Eye drops	Novatears	[24]
		Omega-3 (0.2%) ethyl ester		NovaTears + Omega-3	
				Sodium hyaluronate	
	Hylo Forte				

t0015 **TABLE 15.2** Potential herbal drugs for ocular disease.

S.No.	Scientific name	Common name	Family	Uses	Dosage form	References
1	*Boerhavia Diffusa*	Punarnava	Nyctaginaceae	Night blindness, conjunctivitis	Aqueous distillate	[25]
2	*Sesbania grandiflora*	Hadaga, agati	Fabaceae	Conjunctivitis	in situ gel	[26]
3	*Terminalia chebula, Terminalia bellirica,* and *Phyllanthus emblica,*	Triphala	Combretaceae Combretaceae Euphorbiaceae	Antimicrobial, Antioxidant	Eye drop	[27]
4	*Nigella sativa*	Kalonji	Ranunculaceae	Cataract	Ethanolic extract	[28]
5	*Emblica officinalis*	Amla	Euphorbiaceae		Eye drop	[29]
6	*Ocimum sanctum*	Tulsi	Lamiaceae		Aqueous distillate	
7	*Trigonella foenum*	Fenugreek	Fenugreek		Eye drop	
8	*Cheilanthes glauca*	(Cav.)	Adiantaceae		Eye drop	
11	*Butea Monosperma*	Palash	Fabaceae	Cataract	Eye drop	[30]
12	*Boerhavia Diffusa*	Punarnava	(Nyctaginaceae)	Diabetic retninopathy	Eye drops	[25]
13	*Honey and Rose water*	–	–	Dry eyes	Eye drops	[31]
14	*Boerhavia Diffusa*	Punarnava	(Nyctaginaceae)	Iritis	Eye drops	[25]

they primarily target the anterior section of eye but systemic doses boost the treatment of posterior section of eye. Mostly topical applied drug entities are removed and washed off from the eyeball by mechanism of lacrimation, tear dilution and tear turnover ultimately causing little bioavailability of drugs. Therefore only 5% of administered or topically applied drugs

enters in the eye. The major reasons for the moderate success of existing therapies are little bioavailability of drug after applying to the eyeball [14]. Some of the major limitations of conventional drug delivery system shown in Fig. 15.3 below.

p0255 Some limitations related with dosage forms are discussed below:

s0095 3.1 Eye drops

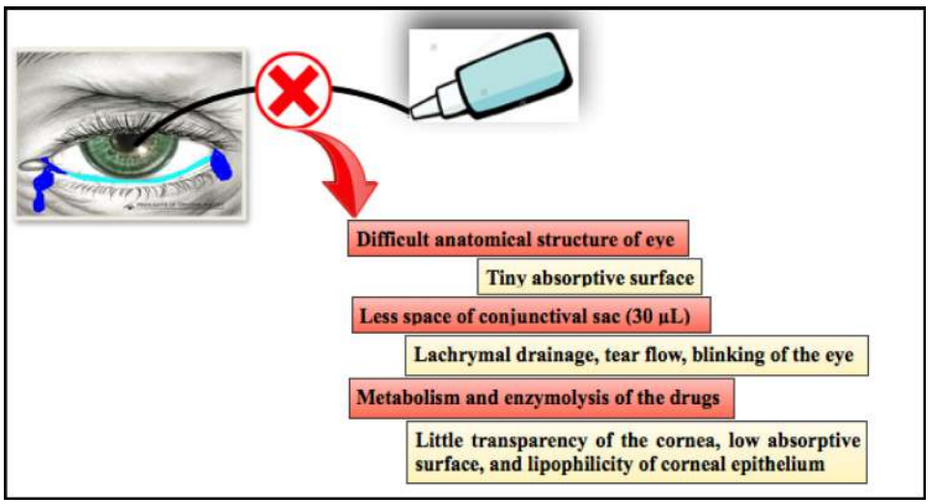
p0260 While using eye drops, some limitations such as lachrymal drainage, tear flow, blinking of the eye, etc. may reduce the quantity of the drug that reaches the retina. Limited capacity of the conjunctival sac, which is approximately 30 μL without blinking. This often leads to spillage of the drug upon administration.

s0100 3.2 Intravitreal injections

p0265 It is an invasive and costly technique. Some side effects include retinal detachment, endophthalmitis, systemic complications like impaired wound healing, hypertension, proteinuria, and increased risk of CVS diseases. Ranibizumab (Lucentis) costs approximately 2150 USD per dose, pegaptanib sodium costs 1000 USD per cost, bevacizumab (Avastin) 55 USD per dose and aflibercept (Eylea) costs €943 per injection.

s0105 3.3 Implants

p0270 Few limitations are there related to implants such as retinal detachment, impaired wound healing.



f0020 FIGURE 15.3 Limitations of conventional drug delivery system.

s0110 3.4 Intravitreal implants

p0275 Dexamethasone has some side effects like cataract and an increase in IOP.

s0115 3.5 Oral, intravenous, subcutaneous or intra-peritoneal routes

p0280 This route is not efficient for delivery of the drug to the eye because only 1%−2% of the drug is able to reach the retina of the eye. There is a decrease in the drug concentration on administration due to various defense mechanisms including tear formation, blinking, and flow of the material via nasolacrimal duct. Metabolism and enzymolysis of the drugs also occur due to the presence of enzymes in the eye.

s0120 3.6 Miscellaneous barriers

p0285 Complex anatomical structure of the eye, little transparency of the cornea, low absorptive surface, and lipophilicity of corneal epithelium are the major reasons for the limited success of conventional dosages forms in several ocular diseases when administered through ocular route [32].

s0125 3.7 Blood retinal barrier (BRB)

p0290 Another major limitation of oral route which does not allow the movement of drugs from blood into the posterior part of the eye. The tight junctions in these cells impede the intercellular permeation. The outer BRBs, limit the drug entry from the choroid into the retina. Although, a systemic administration of drugs is the ideal route for delivery into the retina but BRB stringently modulates drug penetration into retina from the blood. Therefore, for the transportation of drugs from the choroid into the retina, specific/targeted drug delivery systems or different routes of drug administration, for example, suprachoroidal, intraocular, periocular, subretinal, or topical are entailed [20].

p0295 The challenges of the afore mentioned existing conventional dosage forms suggest to rethink on the need for noninvasive, effective, and economical delivery systems. In recent years NDDS, i.e., Novel drug delivery systems have emerged as an effective carrier that can deliver the drug effectively to retina overcoming the challenges associated with conventional as well as surgical approaches. The two main approaches in improving the ocular bioavailability are by prolonging the contact time on ocular surface and increasing the corneal permeability.

s0130 4. Novel drug delivery systems for ocular diseases

p0300 The main aim of NDDS is to reduce the dosing and frequency of injections, increase therapeutic effect, decreases the side effects, improved patient compliance, economic, noninvasive route and overcome the limitations associated with the conventional dosage forms. Different nanocarriers that are proposed for the management of ocular diseases by various researchers are liposomes, solid lipid nanoparticles (SLN), lipid and polymeric nanoparticles, nanostructured lipid carriers (NLC), cationic nanoemulsions, microspheres, prodrugs,

dendrimers, polymeric gels, and ocular inserts etc. Further, advancements of them are discussed below in detail as follows along with the current traditional treatments and advantages of the proposed nanomedicines to target eye [33–35].

s0135 ## 4.1 Liposomes

p0305 Liposome means "lipid body" and their size ranges from 25 to 5000 nm. These are phospholipid vesicles having aqueous part inside having with main advantages such as because of biocompatibility, biodegradability, amphiphilic properties, and relative intoxicity. Drug release from liposomes relies on characters like encapsulation efficiency, size as well as charge of liposomes. Moreover depends upon stability in conjunctival sac and attraction to corneal surface. Normally cell wall consist of lecithin which is natural substance made up by mixture of phospholipids, mainly containing phosphatidylcholine (PC) [36,37]. Hydrophilic drugs can be entrapped and entangled in aqueous (liquid) compartment while hydrophobic drugs can be incorporated in lipid phase and it is shown in Fig. 15.4 [15].

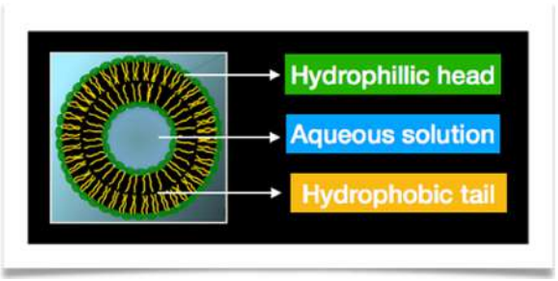
p0310 In one of the studies, rapamycin loaded nanoliposomes was given via a topical route and showed transscleral permeation with high drug retention. This proves effectiveness topical delivery for the management of ocular diseases [38].

p0315 In another study, an artificial virus was used to prepare nanoliposomes for several ocular diseases. Protamine-DNA complex was incorporated inside nanoliposomes for efficient delivery of the Rpe65 gene in the RPe65 knockout mice retina, for treatment of blindness. The results showed significant improvement in vision recovery [39].

p0320 In additional studies, liposomes of citicoline (a well-known drug to treat glaucoma) have been prepared and explored for the treatment of DR [40].

p0325 Nanoparticle of timolol, and timolol maleate chitosan-coated liposomes (TM-CHL) enhanced the precorneal residence time, ocular permeation, bioavailability, and prolonged the residence time on the cornea without any irritation as compared to the commercially available eye drops of TM [41].

p0330 In another study, ranibizumab encapsulated liposomes showed sustained release, greater penetration into the sclera, higher degree of encapsulation, and depot effect as compared to invasive intravitreal injections [42].



f0025 **FIGURE 15.4** Liposome containing hydrophilic as well as hydrophobic drug.

s0140 4.2 Polymeric nanoparticles

p0335 These are microparticles having diameter of below 1 μm, consisting of diverse biodegradable/nonbiodegradable polymers, lipids as well as phospholipids. Nanoparticles (NPs) can easily cross different barriers of the body. Because of the presence of lipids, the permeability of the NPs increases which leads to more retention of the drug in the eye for a longer period. Nanospheres and nanocapsules are its subtypes (Fig. 15.5) based upon whether the drug entity has been homogeneously dispersed or coated within polymeric substance [43].

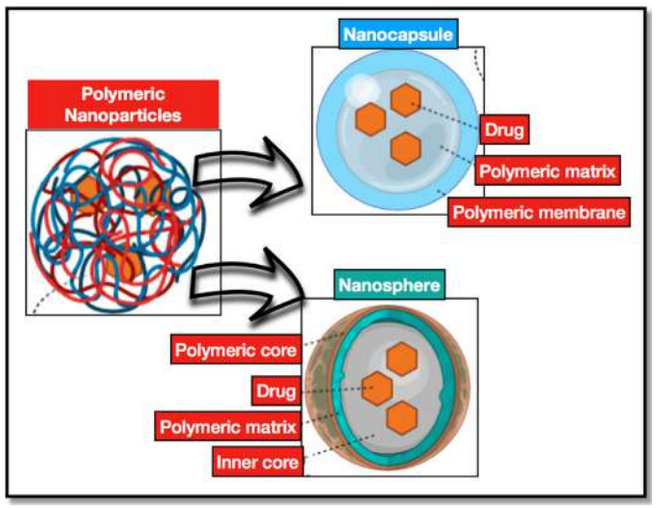
p0340 In the recent studies, NPs were administered intravenously to target the choroid for the management of choroidal neovascularization in the treatment of DR [44]. The neuroprotective role of NPs in DR and delivering the drug to the retina were explored by Kowluru et al. in 2001 [45].

p0345 Another research group focused on the preparation of connexin43 mimetic peptide (Cx43MP) NPs for the treatment of the light-damaged the rodent eye. The prepared NPs exhibit sustained release of drug for the treatment of retinal injury leading to downregulation in inflammation markers, maintaining the retinal structure and function of the eye as compared to the conventional dose as well as reduction of other ocular complications associated with repeated use of intravitreal injections [46].

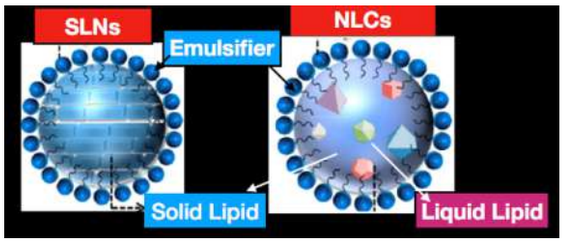
p0350 Lipid NPs are broadly classified as SLNs and NLCs as shown below Fig. 15.6.

s0145 4.3 Solid lipid nanoparticles (SLNs)

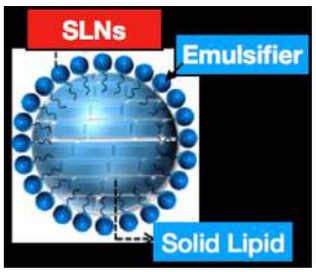
p0355 SLNs are spherical particles with an average diameter between 10 and 1000 nm and provide more surface area, and high drug loading. These formulations consist of solid lipid, surfactant and co-surfactant along with hydrophilic or lipophilic drugs described in Fig. 15.7



f0030 **FIGURE 15.5** Polymeric nanoparticles: Nanocapsule and Nanosphere.



f0035 **FIGURE 15.6** Solid lipid nanoparticles (SLNs) and nanostructured lipid carriers (NLCs).



f0040 **FIGURE 15.7** Solid lipid nanoparticles (SLNs).

below. SLNs are able to achieve the goal of controlled and site-specific drug delivery because of small size, presence of lipids, enhanced stability, excellent biocompatibility, efficient in crossing of biological barriers such as retinal barriers, and increased bioavailability [43,45].

p0360 Triamcinolone acetonide (TA) was explored for the treatment of posterior ocular diseases like uveitis, inflammation, diabetic macular edema and DR. TA-loaded solid lipid nanoparticles (TASLNs) and in situ gel (TA-SLN-IG) formulations were prepared and delivered topically into the deeper ocular tissues [47].

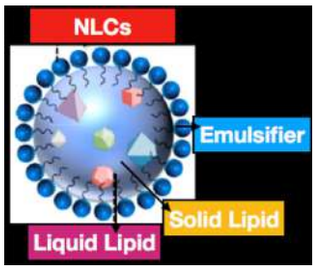
p0365 SLNs of myricitrin improved the antioxidant defense, amount of glycogen, and cellular survival in myotube cells exposed to the hyperglycemic condition [48].

p0370 In another study, SLNs of ciprofloxacin (CIP) were prepared and used for its controlled release to treat ocular diseases. It was concluded that CIP might be administered to remain in contact with the retina for longer time intervals by loading in SLNs [49].

p0375 SLNs have been shown to exhibit lower transfection level in human retinal pigment epithelial established cell line (ARPE-19) and are useful as nonviral vectors for gene therapy for the treatment of retinal disease [50].

s0150 4.4 Nanostructured lipid carriers (NLCs)

p0380 NLCs are drug delivery systems that are generally prepared with both types of lipids, i.e., solids as well as liquids (Fig. 15.8). They can be used topically which can reduce the pain as well as discomfort related to intravitreal injections. Because of the above-mentioned advantages, nowadays NLCs are getting more attention.



f0045 **FIGURE 15.8** Nanostructured lipid nanoparticles (NLCs).

4.4.1 Advantages of NLCs

o0010 **1.** Enhancement of solubility of the hydrophobic drugs in different dosage forms.
o0015 **2.** Ability to enhance the storage stability of different dosage forms.
o0020 **3.** Improvement in permeability and bioavailability of different drugs.
o0025 **4.** Reduction in the adverse effects of some drugs.
o0030 **5.** Prolonged biological half-life of drugs.
o0035 **6.** Targeted delivery of the drug to different tissues in the body can be achieved.
o0040 **7.** Sustained release of drugs achieved in the form of coated NLCs [43].

p0425 Because of the above-mentioned advantages, nowadays NLCs are getting more attention.
p0430 In one of the studies, researchers repositioned itraconazole (ITR) NLCs to manage DR owing to its potent unutilized antiangiogenic effect [51]. In another study, palmitoylethanolamide (PEA) was found to show beneficial effects in several retinal diseases such as DR, glaucoma, etc. PEA attenuated the degree of retinal inflammation while preserving the blood-retinal barrier in diabetic rats [52]. Platania et al. developed Myriocin (Myr) loaded NLCs for the treatment of retinitis pigmentosa (RP) [53]. Table 15.3 having the different examples of drugs for efficient delivery into the posterior segment of the eye.
p0435 Some of the clinical trials ongoing and patents filed for treating ocular diseases are listed in Table 15.4 below.

s0160 5. Conclusion

p0440 The delivery of ocular drugs through the evolving nanocarriers such as nanoparticles, liposomes, SLNs, and NLCs offers promising alternative to overcome the associated challenges with current thera-pies. These nanocarriers provide enhanced drug bioavailability, improved permeation of drug to the retinal areas, enhancement of residence time, noninvasive delivery, better ocular tolerability, etc. Despite these advantages, patient compliance as well as safety always remain a prime concern. Further more research is going on in the area of gene therapy, ocular implant, stem cell therapy as well as laser therapy for the treatment of posterior segment of eye. These advancements offer great benefits in providing the drug in a safer, effective, and more complaint way. In addition to that generation of stable and scalable nanocarrier is always a challenge for the pharmaceutical scientist and industries. Moreover, the availability of these advancements for the clinical use require utmost efforts of interdisciplinary research collaboration.

t0020 TABLE 15.3 Different nano carriers used for ocular diseases.

Novel drug delivery system	Preparation method	Drug	Route of administration	Remarks	References
Liposomes	• Thin lipid film hydration • Extrusion method	Citicoline	Topical administration	• Prevention of glial activation and neural apoptosis in diabetic retina	[40]
	• Film hydration method	Timolol maleate chitosan-coated liposomes	Topical administration	• Enhancement of ocular permeation, precorneal residence time, and bioavailability	[41]
	• Rotary evaporation method	Ranibizumab	Subconjunctival injection to retina	• Higher drug concentration achieved in the retina	[42]
Polymeric nanoparticles (NPs)	• Rotary evaporation method	PEG-PLA chains modified with a cell penetrating peptide (CPP)	Intravenous injection	• Reduces neovascular lesion size • Enhanced drug accumulation	[44]
	• Extensive analysis	Magnetic nanoparticles	Intraocular delivery	• Enhanced drug bioavailability	[45]
	• Emulsification evaporation method	Connexin 43 mimetic peptide	Intravitreal injection	• Reduces possible ocular complications • Preventing the loss of the photoreceptor	[46]
Solid Lipid nanoparticles (SLN)	• Hot homogenization and ultrasonication method	Triamcinolone acetonide	Topical administration	• Drug delivery platform for deeper ocular tissues	[47]
	• Cold homogenization method	Myricitrin	Oral	• Antioxidant • Antiapoptotic • Antidiabetic	[48]
	• Ultrasonic melt-emulsification method	Ciprofloxacin	Intravenous injection	• Controlled release and a superior antibacterial effect	[49]
Nanostructured lipid carriers (NLC)	• High pressure homogenization	Itraconazole	Topical	• Antiangiogenic activity • Improved retention and ocular permeability	[51]

TABLE 15.3 Different nano carriers used for ocular diseases.—cont'd

Novel drug delivery system	Preparation method	Drug	Route of administration	Remarks	References
	• Cold homogenization	Palmitoylethanolamide (PEA)	Topical	• Improved corneal permeability • High ophthalmic tolerability • Prolonged retention capacity • Mucoadhesive and film forming properties	[52]

t0025 TABLE 15.4 List of clinical trials ongoing and patents filed for ocular diseases.

S. no	Name of clinical trial/patent	Outcome	Clinical trial no/ patent no	Year	Reference
1	Imaging of uveitis patients receiving injectable fluocinolone acetonide implant	Prospective imaging of the intravitreal fluocinolone acetonide implant using fluorescein angiography and optical coherencE tomography in uveitis patients	NCT04340505 (clinical trail)	2021	[54]
2	Study to gather information on safety and use of high dose aflibercept injection into the eye in patients with an age-related eye disorder that causes blurred vision or a blind spot due to abnormal blood vessels that leak fluid into the light sensitive lining inside the eye	Changes in visual acuity (clarity of vision) with a high dose treatment with aflibercept (Eylea) in patients suffering from neovascular age-related macular degeneration (nAMD)	NCT04423718 (clinical trail)	2021	[55]
3	Effect of traditional Chinese Medicine on basic tear secretion and tear cytokines in patients with dry Eye disease	Explore the efficacy of traditional Chinese Medicine for dry eye disease	NCT04785261 (clinical trail)	2021	[56]
4	Visual outcomes with a trifocal IOL in subjects with open-angle glaucoma	Cataract surgery with implantation of trifocal IOL combined with trabecular scaffold	NCT04619654 (clinical trail)	2021	[57]
5	Study of the correlation between preoperative precise biometrics spatial assessment and postoperative visual quality in cataract patients	Summarizes the experience of precision treatment and postoperative management of various types of cataract	NCT04833491 (clinical trail)	2021	[58]
6	Gene therapy for eye pathologies	Compositions and methods are described for the delivery of therapeutic products to the retina/ vitreal humor in the eyes of human subjects to treat pathologies of the eye	WO2020206098A1 (patent)	2020	[59]

(*Continued*)

TABLE 15.4 List of clinical trials ongoing and patents filed for ocular diseases.—cont'd

S. no	Name of clinical trial/patent	Outcome	Clinical trial no/ patent no	Year	Reference
7	Modified aav8 capsid for gene transfer for retinal therapies	A method of preventing, arresting progression of, or ameliorating vision loss associated with an ocular disorder in a subject	EP2954051B1 (patent)	2019	[60]

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Non-Print Items

Abstract

The topical route is the most common route for delivery of the drug to the eyes because of its high patient compliance and noninvasive technique. Mostly, the drug is not able to cross the barriers and is unable to reach the posterior eye segment. Success of any treatment of ocular disease depends on the targeting of the drug to the posterior segment of eye, i.e., retina by crossing or bypassing ocular barriers for the management of ocular diseases. In particular, ocular diseases have been treated by two primary modalities, i.e., topical drops and intravitreal injections. But they can cause serious complications of eyes like impaired wound healing, retinal detachment, endophthalmitis, hypertension, proteinuria, and increased risk of cardiovascular diseases. Ocular conventional systems include ophthalmic drops, eye suspensions, eye emulsions, and eye ointments are not considered most advantageous for treatment of ocular diseases as they primarily target the anterior section of eye but systemic doses boost the treatment of posterior section of eye. Mostly topically applied drug entities are removed and washed off from the eyeball by mechanism of lacrimation, tear dilution, and tear turnover ultimately causing little bioavailability of drugs. The challenges of the aforementioned existing conventional dosage forms suggest to rethink on the need for noninvasive, effective, and economical delivery systems, i.e., novel drug delivery systems. They can deliver the drug effectively to retina overcoming the challenges associated with conventional approaches.

Keywords:

Intraocular delivery; Noninvasive techniques; Novel drug delivery systems.

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Review Article

Multifaceted role of synbiotics as nutraceuticals, therapeutics and carrier for drug delivery

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ABSTRACT

Synbiotics, are a combination of probiotics and prebiotics. They play an important role in metabolizing different nutritional substrates and thus helps in the maintenance of human health. Any disbalance in the gut microflora, known as dysbiosis, is known to lead to a number of diseased conditions. It can be reverted by the administration of synbiotics. Present review highlights various mechanistic pathways through which synbiotics act as therapeutics. The dual role of synbiotics as nutraceutical and excipient in developing oral formulations are entailed with case studies. The findings entailed that there exist numerous studies on prebiotics as well as probiotics have been carried out to show their effects in several diseases. However, the concept of combining together them for prevention and treatment of various pathological conditions accruing from dysbiosis is relatively new. Synbiotics, however, face challenge of low stability during their sojourn in the GIT, which is generally overcome by various encapsulation techniques. Various studies also showed potential role of synbiotics in drug delivery. However, it is an emerging area and lacks clinical correlation. It is important to focus on clinical trials of formulations wherein synbiotics have been used as therapeutic moiety as well as pharmaceutical carrier for treating various diseases.

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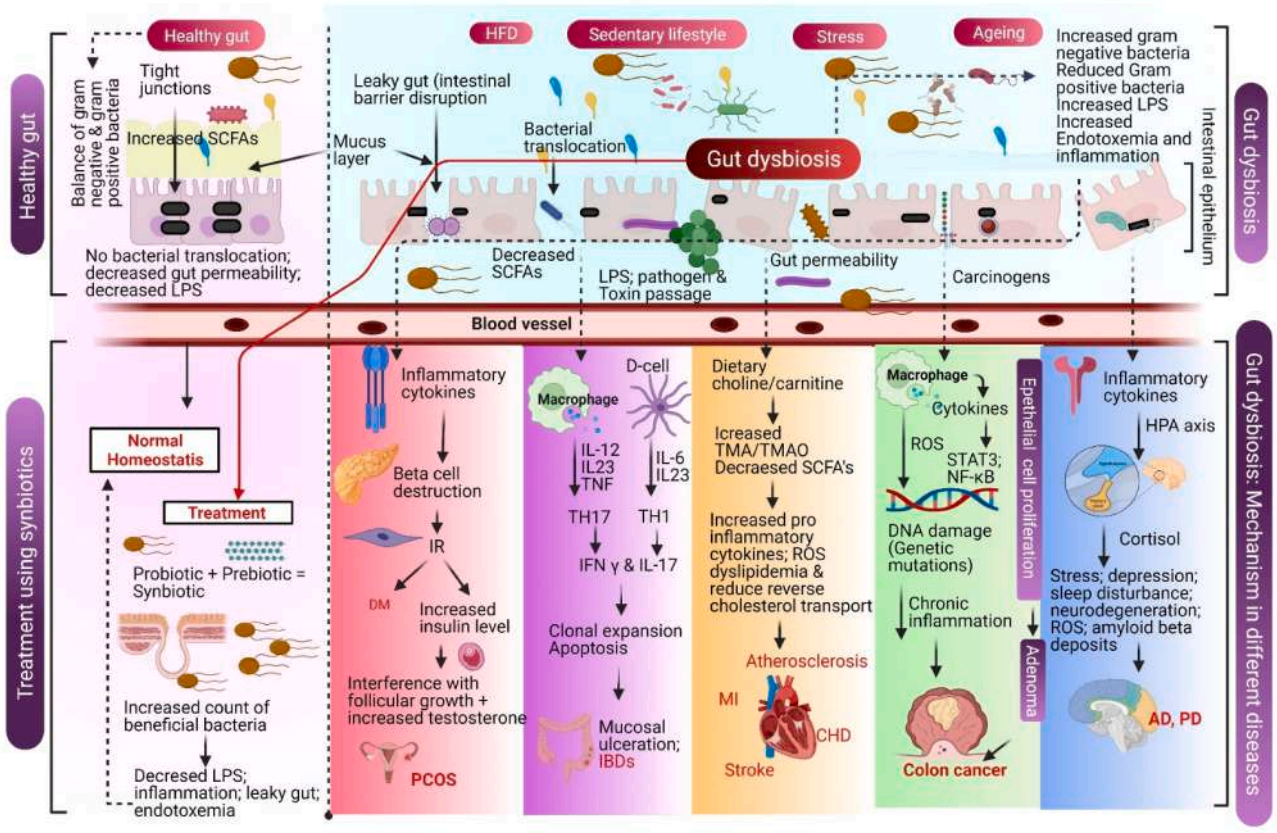

Fig. 1. Impact of gut dysbiosis in the pathogenesis of diseases.

1. Introduction

Prebiotics and probiotics have emerged as an attractive functional food in last three decades. A lot of research has been carried out to decipher the role of microbiome in various pathological states. Realization of the significance of eubiosis in combating various diseases has led to wider acceptance of prebiotics and probiotics [1], with a projected 7% annual growth of probiotic industry [2], and an estimated 12.7% growth of prebiotic industry over the next eight years [3]. Despite an overall consumer opinion that prebiotics and probiotics are useful in prevention as well as treatment of diseases, a gap still exists in understanding their health benefits, their mechanisms of action in the treatment of various disorders, and even definitions of the terms [1,4].

The word "Probiotic" which means "for life" defines the living non-infective bacteria present in the gut that exert useful effects on hosts [5]. The Food and Agriculture Organization (FAO, 2002), defines probiotics as "live microorganisms which when administered in adequate amounts confer a health benefit to the host" [6]. Lactic acid bacteria such as *Lactobacilli* and *Bifidobacteria* are the most common probiotics which are commercialized. These are mostly supplemented in fermented dairy foods and are also available in market in their lyophilized forms.

The components of food which cannot be digested in the gastrointestinal tract (GIT) and support the growth of useful gut bacteria, thereby conferring various therapeutic effects to the host, are termed as prebiotics. This term was coined in 2004, according to which prebiotics were described as selectively fermented components which permit certain alterations in the activity and/or composition of bacteria present in the GIT, thereby bestowing various useful effects. Later, WHO/FDA in 2007 defined prebiotics as the non-living food components providing health benefits to the host by modulating the indigenous microflora [5,6]. Prebiotics stimulate the growth of probiotics and can help in modifying the gut microbial composition and restoring eubiosis.

Gibson and Roberfroid in 1995, coined the term "Synbiotics" to define the synergistic actions of probiotics and prebiotics [5]. Since the term "synbiotics" refers to synergy, the term is used only for such food items in which the development and growth of probiotic strain are significantly supported by the prebiotic. Synbiotics are formulated to ensure the survival and growth of probiotic strains. Consequently, a satisfactory blend of both the components must deliver more health benefits in comparison to those by each component when administered individually. Synbiotics are commonly found in fermented and dairy products, fruits, and raw vegetables [6]. Enhancement in the count of useful microbes and the decrease of the pathogen load has been established after synbiotics supplementation in farm animals [7]. In spite of the fact that the role of synbiotics has been proven, significant data relating to their influence on the host is so far inadequate [8]. The health effects of synbiotics have been explored in a number of diseases such as diabetes mellitus (DM) [9], cancer [10], neurodegenerative diseases (NDs) [11], cardiovascular diseases (CVDs) [12], and inflammatory bowel diseases (IBDs) [13].

In the current review, we elaborate the mechanisms of action of synbiotics in the treatment of various diseases while discussing the research carried out on synbiotics as therapeutic agents either alone or in combination with other formulations. Also discussed are the effects of co-administration of synbiotics along with different drugs on their release as well as therapeutic efficacy. The commercialization of synbiotics has been discussed along with enlisting the marketed products, and clinical trials that have been conducted so far.

2. Gut microbiota and dysbiosis

The term "microbiota," or "microflora," refers to bacterial, viral, helminthic and rickettsia species coexisting in the human gut of the host [14]. About 10^{14} bacterial cells are reported to be found in human

Table 1
Table depicting the studies carried on animals and humans to show therapeutic efficacy of synbiotics in various diseases.

S. N.	Synbiotic composition and dose	Animal study (model)	Human study (Trial; No of participants)	Treatment duration	Disease	Results	Reference
1.	Synbiotic capsule (*L. casei* and *B. bifidum* (2×10^9 CFU/g each) + 800 mg inulin	NA	R DB PC 27	8 weeks	Rheumatoid arthritis	Decreased hs-CRP; Increased NO; reduced HOMA-IR and HOMA-B; increased GSH	[191]
2.	Immunofortis® (*B. breve* 10^{10} CFU + long-chain FOS and short-chain GOS in the ratio of 1:9)	NA	R PC DB 29	4 weeks	Allergic reaction in asthmatic patients	Reduced Th2-cytokines production; improved PEF; inhibited IL-5 synthesis	[192].
3.	Yakult BL Seichoyaku (*B. breve* and *L. casei* 1×10^8 CFU each + GOS (10 g/day)	NA	R C SB 35	4 year 10 months	Sepsis	Increased urine *Lactobacillus* and *Bifidobacterium* and total organic acid concentration, particularly the amounts of acetate	[193]
5.	Yakult BL Seichoyaku (*B. breve* and *L. casei* 1×10^8 CFU each + GOS (12 g/day)	NA	R PC 21	14 weeks	Biliary cancer: Postoperative infections	Reduced harmful microorganisms; increased total organic acid concentrations; reduced infectious complications incidence in synbiotics group (19%) compared to controls (52%)	[194]
6.	Synbiotic capsules (10^9 CFU/g of 7 probiotic strains including *L. bulgaricus, L. acidophilus, L. rhamnosus, L. casei, B. longum, B. breve, and S. thermophiles* + 38.5 mg FOS	NA	R PC 45	10 weeks	Breast cancer-related lymphedema	Reduced total quality of life score, and it's psychosocial and functional domain scores, reduced edema volume and BMI	[10]
7.	*Synbiotic capsule containing 200 million bacteria (L. rhamnosus, B. breve, S. thermophilus, B. longum, L. casei, L. acidophilus,* and *L. bulgaricus)* + 125 mg FOS	NA	DB R PC 50	28 weeks	NAFLD	Reduced hepatic steatosis and fibrosis; reduced BGL, and TAG	[195]
8.	*L. plantarum, L. acidophilus* 2×10^9 CFU/dose each and *L. reuteri* 6×10^9 CFU/dose + inulin and FOS (12.97% each)	NA	60 R PC	2 months	Metabolic syndrome	Improved waist circumference, fasting plasma insulin, TC, HDL-C, TG, hs-CRP, TNF-α; reduced mean arterial pressure and BGL	[196]
9.	*L. rhamnosus* and *L.delbrueckii* + XOS 0.2 g/kg and red ginseng extracts	Mice	NA	20 days	Dysbiosis	Reduced the ratio of *Firmicutes/Bacteroidetes*, inhibited injurious bacteria (e.g. *Escherichia coli*), and hastened the regaining of useful bacteria (e.g., *Lactobacillus*); prolonged colonization time; increased intestinal bacterial population	[18]
10.	10^9 CFU of probiotics combination of Bifidobacterium, Lactobacillus and Streptococcus + inulin 1 g/kg	SD rat	NA	5 weeks	CKD	Enhanced the profusion of indole-synthesizing bacteria: Prevotella, Clostridium and Bacteroides	[197]
11.	Synbiotic capsules containing seven strains of probiotic bacteria (2×10^8 CFU) + FOS as a prebiotic; 2 capsules/day	NA	R C TB 46		Metabolic syndrome	Improved body weight, BMI, BGL, insulin, HOMA-IR, and GLP-1; increased peptide YY	[198]
12.	300 g synbiotic yogurt containing 10^8 CFU *B. animalis* + 1.5 g inulin	NA	R C OL	24 weeks	NAFLD	Improved hepatic steatosis and concentration of enzymes (AST, ALT, ALP)	[199]
13.	*Leuconostoc mesenteroides, L. plantarum* and *Pediacoccus pentoseceus*, (10^{10} CFU) each + 10 g fermentable fiber (inulin, beta glucan, resistant starch, and pectin 2.5 g each)	NA	R 20	30 days	MHE	Reduced gut dysbiosis, endotoxemia & blood ammonia levels; reversed MHE; increased *Lactobacillus* species in urine	[200]
14.	3 g/kg synbiotics (Bioture, consisting of *Saccharomyces cerevisiae, Bacillus subtilis* + β-glucan and mannan oligosaccharide)	Pacific white shrimp	NA	56 days	Non-specific immunity	Increased weight gain; hepatopancreatic protease activity, levels of SOD, CAT, ALP; reduced MDA; increased *Lactococcus* abundance and reduced *Vibro* abundance	[201]
15.	Lactococcus lactis 1000 CFU + trans GOS 0.528 mg	Broiler	NA		Immunity	Stimulated immune system (an increase in B splenocytes and a decrease in T splenocytes were observed)	[202]
16.	*B. animalis* 10 billion CFU/day + FOS 4 g twice/day	NA	DB 104	10–14 months	NAFLD	Fecal microbiomes of synbiotic group had increased count of *Faecalibacterium and Bifidobacterium* species, and decreased count of *Oscillibacter*	[203]
17.	L. plantarum, L. reuteri, L. pentosus, L. rhamnosus, L. paracasei, *S. cerevisiae* + inulin	Turkey	NA	15 weeks	Performance	Increased useful bacteria and reduced pathogen in the digestive tract. Increased α-galactosidase and α-glucosidase activity	[204]
18.	10^8–10^9 CFU each of L. paracasei, L. rhamnosus, L. acidophilus and B. lactis +6 g FOS	NA	Pilot study 9	3 months	Inflammation	Preserved hydration status	[205]
19.	*B. breve* 15 mg, *L. casei* 20 mg, + GOS 15 g/day	NA	R 50	2 weeks	Infectious complications	Reduced infectious complications	[206]

(*continued on next page*)

Table 1 (*continued*)

S. N.	Synbiotic composition and dose	Animal study (model)	Human study (Trial; No of participants)	Treatment duration	Disease	Results	Reference
20.	*B. bifidum, B. lactis, L. acidophilus,* and *B. longum* (1.5×10^9 each) + inulin	NA	R DB PC PG 120	24 weeks	Prediabetes	Reduced hyperglycaemia, hypertension and HDL-C	[207]
21.	Nine different strains of *Bifidobacteria, Lactobacillus*, and *Streptococcus* (45 billion CFU) + Prebiotics 7.5 g daily (inulin high performance, FOS, and GOS	NA	R DB PC CO	6 weeks	Renal failure	Reduced p-cresyl sulfate; altered the stool microbiome, increased *Bifidobacterium* and reduced *Ruminococcaceae abundance*	[208]
22.	*L. paracasei* 2.5×10^7 CFU/100 g of body weight + arabinogalactan and FOS 5 and 7 mg/100 g respectively	Rats	NA	6 weeks	NAFLD	Reduced liver inflammatory markers; increased expression of nuclear PPAR and expression of downstream target genes; Reduced IR, and improved hormonal homeostasis and glycaemic control; protected damage of hepatic insulin signalling; maintained gut barrier integrity and decreased the relative count of Gram-negative *Escherichia coli* and *Enterobacteriales* in colon	[209]
23.	Synbiotics capsules (*L. acidophilus* 7×10^9 CFU, *L. bulgaricus* 2×10^8 CFU, *B. breve* 2×10^{10} CFU, *L. rhamnosus* 1.5×10^9 CFU, *B. longum* 7×10^9 CFU, *S. thermophilus* 1.5×10^{10} CFU, *L. casei* 2×10^9 CFU,+ FOS 500 mg/day)	NA	R DB PC 30	8 weeks	Hypothyroidism	Elevated CRP; may have beneficial effects on thyroid function	[210]
24.	2 Familact capsules daily each 500 mg (*L. acidophilus, L. bulgarigus, L. rhamnosus, B. longum, L. casei, B. breve,* + FOS)	NA	R C 66	6 weeks	Azotemia in CKD	Decreased blood urea nitrogen in CKD patients	[211]
25.	Protexin capsule	NA	R C 66	4 weeks	Constipation	Increased stool frequency	[212]
26.	(FloraGuard®) *L. acidophilus* and *L. bulgaricus* (5.4×10^8 CFU), *S. thermophilus* (1.2×10^8 CFU), *B. bifidum and B. longum* (1.3×10^9 CFU each), Proprietary blend culture count (2×10^9) CFU + inulin 50 mg	Rats	NA	12 weeks	Ethanol induced liver injury	Reduced hepatic TG, plasma endotoxin, and TNF-α levels, Increased IL-10 level protected the rats against hyperpermeability of the intestine induced by ethanol, increased abundance of faecal *Bifidobacteria*	[213]
27.	*B. breve* 2×10^9 CFU + non digestible oligosaccharides	Mice	NA	2 weeks	Allergic inflammation	Decreased IL-6, IFN-γ, and IL-10 levels	[214]
28.	*Pediococcus acidilactici* + GOS	rainbow trout	NA	8 weeks	Innate immune response	Increased immune response	[215]

ALT: Alanine transaminase; AST: aspartate transaminase; ALP: Alkaline phosphatase; BGL: Blood glucose level; CAT; Catalase; CFU: Colony forming unit; C: Control; CO: Crossover; CKD: Chronic kidney disease; DAS: Disease activity score; DB: Doble blind;FOS: Fructooligosaccharide; GIT; Gastro intestinal tract; GOS: Glactooligosaccharide; IL-6: Interleukin-6; IFN- γ: Interferon gamma; MHE: Minimal hepatic encephalopathy; NAFLD: Non-alcoholic fatty liver disease; NO: Nitrogen oxide; OL: Open label; PC: Parallel control; PG; Parallel group; PEF: Peak expiratory flow; QR: Quadruple; R: Randomized; SCFAs: Short chain fatty acids; SB: Single blind; SOD: Superoxide dismutase; TAG: Triacylglycerol; T2DM: TB: Triple blind; TH2: T-helper cell 2; VAS: Visual analogue scale.
*B: Bifidobacterium * L: Lactobaccilus; *S: Streptococcus.

microbiome [15]. These microbes are mostly colonized in the GIT; 70% of the total human microflora is housed in the colon alone [15]. Any dysbiosis in the gut microbiota plays a significant role in the development of a number of diseases. GIT mucosa is continuously exposed to an environment which is rich in antigens of bacterial origin and food particles. Some of the functions of gut microbiota include maintenance of function as well as structural integrity of intestine, regulation of growth and development of tissues and cells, prevention of pathogen colonization, immunomodulation, and performance of numerous metabolic activities important for the host's metabolism [16]. So, any significant alteration in the composition of gut microbiome may lead to an alteration in any of these, eventually leading to a diseased condition.

The intestinal milieu comprising of digestive enzymes, gastric acid and immune globulin A (IgA) creates a defensive barrier and is detrimental to invasive and swallowed infective and pathogenic microbes. The commensal microbes damage intraluminal antigens and prevent the adherence and colonization of infective microbes. They also play a significant part in the initiation of regulatory T cells [17]. The association between gut dysbiosis and development of certain diseases such as DM [9], cancer [10], and IBDs [13] has been reported widely. The role of dysbiosis in development of these diseases is presented in Fig. 1.

Synbiotics have been effectively used to restore gut eubiosis; thus, contributing towards management of various diseases. These, therefore, also influence the interactions of microbiota with the gut epithelium as well as the immune system [18]. Although probiotics and prebiotics alone can exert beneficial effects in host, but there are a number of studies that have reported a more significant effect of synbiotics than those of either of their components. For example, in one of the studies, it was seen that when probiotics and prebiotics (+β-(1,3/1,6)-glucan derived from *Schizophyllum commune*) were administered alone and in combination, the synbiotics as well as probiotic group led to a greater diversity of probiotic strains in the gut, while prebiotic administration only, enhanced the overall richness and host immune strengthening. The group treated with probiotics also resulted in decreased metabolic activities due to short chain fatty acid (SCFA) synthesis, which were not decreased in prebiotics and synbiotics treatment groups. The synbiotics showed higher potential than either of their two components used individually [19]. In another study, it was found that inulin and fructooligosaccharides (FOS) remarkably increased viability and prolonged the retention period of *Lactobacillus acidophilus*, *Bifidobacterium lactis* and *L. casei* [20]. One more study by Adebola and coworkers indicated that prebiotics, lactobionic acid and lactulose enhanced the tolerance of *L. acidophilus* and *L. reuteri* to taurocholic and cholic acid remarkably. The authors recommended that prebiotics may act as a substitute of energy source for *Lactobacilli* which facilitate them to react better to the bile acid stress [21]. Gopal and coworkers [22] reported that dietary

Table 2
Table exclusively indicating the ongoing and completed clinical trials that have been explored to clinically evaluate the therapeutic potential of synbiotics in treating various diseases [216].

S. N.	Status	Title	Conditions	Phase	Design	Outcome measures	Number of subjects	Study start date	Study end date
1.	Unknown	Synbiotics and low-grade inflammation in obese subjects	T2DM, obesity, IR	NA	R PD	LPS binding protein, IL-6	44	Nov 2010	Jan 2011
2.	Enrolling by invitation	Clinical study on the effect of a synbiotic on body fat mass	T2DM, obesity	NA	R PD	HOMA-IR, HbA1c	120	May 2020	Dec 2021
3.	NRY	Study to determine the effect of synbiotics in patients with pre-diabetes	Prediabetes	NA	R PD TB	BGL	60	March 2021	Dec 2022
4.	NRY	The effect of synbiotic consumption on glycemic, inflammatory markers and body composition on prediabetic and diabetic patients	Dietary supplements	3	R PD TB	Glycaemic and inflammatory markers	160	Oct 2020	June 2021
5.	Completed	Synbiotic therapy on intestinal microbiota and insulin resistance in obesity	Obesity, IR	4	R PD QR	HOMA-IR	16	Sep 2019	Dec 2020
6.	Completed	Mechanism of microbiome-induced insulin resistance in humans (aim2)	Insulin sensitivity	2	R PD TB	Insulin sensitivity, gut permeability, endotoxin level	20	April 2014	Dec 2019
7.	Completed	The effect of a bifidobacterium and polydextrose on body fat mass	Obesity, IR, DM	2	R PD QR	Change in BMI and weight	225	Nov 2013	May 2015
8.	Completed	Effects of synbiotic supplementation in metabolic syndrome	Metabolic syndrome	2 3	R PD QR	HOMA-IR	38	April 2012	–
9.	Unknown	Effect of synbiotic supplement on the body mass index of participants with severe obesity	Obesity class 3	NA	R PD QR	BMI, CRP, TC	180	September 2017	July 2018
10.	NRY	Effects of synbiotics supplementation on the uremic toxin indoxyl sulfate level and constipation in end-stage renal disease patients undergoing haemodialysis	Constipation, end stage renal disease	NA	R PD DB	Indoxyl Sulfate concentration, constipation symptoms and related life quality	60	Sep 2020	Feb 2021
11.	Completed	Synbiotics and gastrointestinal function related quality of life after colectomy for cancer	Colorectal neoplasms	NA	R SG QR	Postoperative evaluation of GIT function-related life quality	100	Nov 2010	Sep 2015
12.	Unknown	Effect of synbiotic on postoperative complications after liver transplantation	Liver disease	NA	R PD DB	Antibiotic therapy duration, postoperative infection, postoperative duration of hospital stay	76	March 2016	Jan 2018
13.	NRY	Synbiotic compound to reduce symptoms of schizophrenia	Schizophrenia	2	R PD QR	Inflammatory markers, PANSS, MCCB and MATRICS changes	68	July 2023	July 2023
14.	NRY	Synbiotics and post-op Crohn's disease	Crohn's disease	NA	R PD	Changes in life quality	36	April 2021	June 2023
15.	Completed	The effects of synbiotics supplement on biochemical factors and hepatic fibrosis in patients with non-alcoholic steatohepatitis	Non-alcoholic steatohepatitis	2 3	R PD QR	ALT, Liver fibrosis, BMI	54	March 2012	Jan 2013
16.	Completed	Safety of synbiotics as adjuvant to influenza vaccine in elderly	Influenza	1	R PD QR	Immune response to vaccine, compare probiotic presence in stool	22	Dec 2010	April 2011
17.	Unknown	Effect of a synbiotic supplement on a high-protein diet	Obesity	NA	R	Gut microbiota alterations; BGL and body weight	40	March 2017	Nov 2017
18.	Completed	Pilot trial of a synbiotic in HIV positive patients	HIV infection	NA	R PD TB	LPS, immune activation	34	May 2008	Nov 2009
19.	Completed	Effect of synbiotic on immune response, gut permeability and microbiota in patient with connective tissue disease	Connective tissue disease	NA	R PD DB	FOXP3 regulatory T cell, IL-17, zonulin, gut microbiota	46	Oct 2017	Aug 2018

(*continued on next page*)

Table 2 (*continued*)

S. N.	Status	Title	Conditions	Phase	Design	Outcome measures	Number of subjects	Study start date	Study end date
20.	Completed	Impact of a synbiotics containing fructo-oligosaccharides and *bifidobacteria* in middle-aged adults	Constipation	NA	R PD QR	Intestinal transit, stool consistency, evaluation of life quality	27	June 2016	Jan 2019
21.	Recruiting	Effect of synbiotics 365 on body composition in overweight and obese individuals	Obesity	NA	R PD TB	Change in BMI, Body weight, central obesity	180	Sep 2020	Sept 2021
22.	Completed	Investigation of synbiotics treatment in NAFLD	NAFLD	NA	R PD DB	Changes in liver fat, Bifidobacterium species, NAFLD and enhanced liver fibrosis score	104	Dec 2013	Jan 2019
23.	Completed	How does a synbiotics supplement affect iron status during iron repletion in iron depleted female athletes?	Iron deficiency	NA	R PD DB	Iron status	20	Aug 2016	Dec 2017
24.	Completed	Synbiotics treatment in Corhn's disease patients	Crohn's disease	NA	R SG DB	Decrease in mucosal TNF-α, differences in TNF-α, IL-18 and IFN-gamma before and after synbiotics treatment	50	June 2006	Dec 2008
25.	Terminated	The role of synbiotics in reducing post-operative infections in patients undergoing cardiac surgery: a pilot study	Surgical wound infection, cystitis, bacteraemia	4	R PD QR	Infection, tolerance	40	Nov 2005	June 2007
26.	Recruiting	An evaluation of a synbiotics formula for patients with covid-19 infection	Covid19	NA	R PD OL	Clinical improvement, time to develop antibody against COVID	50	Aug 2020	April 2022
27.	Completed	Effect of the synbiotics probinul-neutro® on gastrointestinal symptoms and plasma p-cresol level in chronic renal failure	CKD	4	R DB PD	Changes in plasma p-cresol concentration, composite outcome of GIT symptoms	30	April 2013	April 2013
28.	Completed	The effects of a synbiotics addition on eradication therapy of helicobacter pylori infection in children	*Helicobacter pylori* infection	NA	R PD OL	The eradication rates, eradication therapy related side effects	69	June 2011	Aug 2012
29.	Completed	Effect of prebiotic and synbiotics supplementation in individuals undergoing roux-en-y gastric bypass.	Obesity	NA	R PD DB	Change in IL-1, IL-6 and TNF-α concentrations	18	Oct 2013	July 2015
30.	Completed	Action of synbiotics on irradiated GI mucosa in rectal cancer treatment	Rectal cancer	NA	R PD DB	Action of synbiotics on irradiated GI mucosa in rectal cancer treatment	30	Nov 2008	November 2015
31.	Completed	Effect of prebiotic or synbiotics on inflammatory response and indicators of nutritional status in obesity	Morbid obesity	NA	R PD QR	CRP, cytokines, TGs	22	Jan 2016	April 2018
32.	Completed	Synbiotics in infants with cyanotic congenital heart disease	CHD	NA	R PD QR	Sepsis, necrotizing enterocolitis	100	Oct 2012	May 2013
33.	Completed	The effect of a prebiotic, probiotic and synbiotics on the gut microbiota and immune response of older volunteers	Gut microbiota, immune function	NA	R CO TB	Changes to the gut microbiota, immune function	50	March 2008	Oct 2009
34.	Completed	Synbiotics treatment of ulcerative colitis patients	UC	NA	R SG TB	Decrease in mucosal TNF-α, induction of clinical remission calculated by a decrease in Mayo disease activity score	24	Jan 2009	Jan 2011
35.	Unknown	Prevention of febrile neutropenia by synbiotics in paediatric cancer patients	Febrile neutropenia, infection in an immunocompromised host, cancer	2	R PD QR	Decrease in the febrile neutropenia episode incidence, safety evaluated according to CTCAE Version 4.0, reduction of the total duration of febrile neutropenia	120	July 2010	Oct 2019
36.	Completed	Significance of synbiotics on inflammation and	Inflammation	NA	R PD DB	Reactivity of rectal mucosa, influence on immune system	12	Jan 2006	Feb 2012

(*continued on next page*)

Table 2 (*continued*)

S. N.	Status	Title	Conditions	Phase	Design	Outcome measures	Number of subjects	Study start date	Study end date
		proliferation of colonic mucosa							
37.	Recruiting	Evaluation of a synbiotics formula in patient with covid-19	COVID	NA	R PD QR	Changes in gut microbiome, faecal bacteria metabolites, and plasma cytokines including IL-6, TNF-α and CXCL-10	20	Aug 2020	March 2022
38.	Completed	Effect of synbiotics on composition of human gut microbiota and production of short and branched-chain fatty acids	Synbiotics, healthy humans, composition of gut microbiota, SCFAs	Early phase 1	R CO TB	Enhancement in faecal butyrate concentration, Alterations in gut microbiota concentration and SCFAs	18	March 2011	June 2011
39.	Completed	Effect of starter formula on infection prevention	Gastro-intestinal infections, infections with fever	NA	R PD QR	Diarrhoea and all infections with fever, morbidity, anthropometry	477	Oct 2008	Oct 2013
40.	Completed	Use of a novel synbiotics to change human gut bacteria and improve health in obese adults	Intestinal microbiota and barrier function	NA	R PD DB	Changes in microbiota, intestinal permeability and endotoxemia	151	June 2013	Oct 2015
41.	Recruiting	Study to determine the effect of synbiotics in patients with pre-diabetes	Prediabetes	NA	R PD DB	BGL	60	April 2021	Dec 2022
42.	Completed	Impact of symbiotic administration on intestinal function of head and neck patients surgically treated	Head and neck cancer	NA	R PD TB	Serum DAO enzyme concentration, infection rate	40	Oct 2014	July 2016
43.	Unknown	Effect of short-term synbiotics treatment on plasma p-cresol levels in haemodialysis patients	Haemodialysis	4	R PD DB	Changes in plasma p-cresol concentration, composite outcome of GIT symptoms	30	Dec 2014	Feb 2015
44.	Completed	A study of a prebiotic, a probiotic and a synbiotics upon the gut microbiota and immune response of healthy volunteers	Gut Microbiota, bowel function, immune function, plasma lipids	NA	R CO TB	Changes to the gut microbiota, bowel function, immune function and plasma lipids	44	Jan 2008	Jan 2010
45.	Recruiting	Synbiotics therapy of gastrointestinal symptoms during covid-19 infection	COVID	NA	R QR PD	Stool calprotectin, frequency and consistency	120	Sep 2020	Dec 2022

COVID: Coronavirus disease; CTCAE: Common terminology criteria for adverse events; CXCL-10: C-X-C motif chemokine ligand 10; DAO: Diamine oxidase; FOXP3: Forkhead box P3; HIV: Human immunodeficiency virus; MATRICS: measurement and treatment research to improve cognition in schizophrenia; MCCM: MATRICS Consensus Cognitive Battery; NRY: Not yet recruiting; PANSS: Positive and negative feedback scale; PD: Parallel design; SG: Single group.

intake of milk with synbiotics containing oligosaccharides and *B. lactis* enhanced the abundance of *Bifidobacteria* and *Lactobacilli* in the human GIT. In one of the randomized, double blind (DB) and crossover trial, 30 healthy children (14 girls and 16 boys) were administered probiotic alone and synbiotics for 3 weeks after which their intestinal microflora was compared. It was seen that intake of 65 mL of synbiotics fruit juice containing *L. rhamnosus* and galactooligosaccharides (GOS) resulted in a remarkable increase of *Bifidobacteria* compared to the group administered with probiotic alone [23]. In case of treatment of certain ailments including colon cancer and ulcerative colitis (UC), administration of synbiotics showed a pronounced effect as compared to those of therapies that were restricted to prebiotics or probiotics alone [24,25]. These outcomes reveal that synbiotics seem to be more beneficial than using either of the components alone. Some more studies describing the effect of synbiotics for different conditions are discussed in subsequent sections and listed in Table 1. The different clinical trials which are either completed or are undergoing have been summarized in Table 2.

2.1. Composition of gut microbiota

There is different kingdom of microorganisms (e.g. viruses, fungi and bacteria) which make up the composition of gut microbiota. The previously reported studies indicated that there are more than 1000 species of microbes belonging to different phyla are present in gut microbiota. However, among them only a small number of phyla are well characterized, which include only 160 species [26]. The most prominent phyla present in the gut include Actinobacteria, Firmicutes, Bacteroidetes, Actinobacteria, Verrucomicrobia, Proteobacteria and Fusobacteria. Among these, 90% of the gut microbiota is composed of Firmicutes and Bacteroidetes [27]. Further there are almost 200 different genera (*Bacillus*, *Enterococcus, Clostridium*, *Ruminicoccus and Lactobacillus*) which make up the composition of Firmicutes phylum. Among these genera, *Clostridium* genera represent 95% of the Firmicutes phyla. The predominant genera present in Bacteroidetes includes *Bacteroides* and *Prevotella*. The phylum Actinobacteria is comparably less abundant and mostly represented by the genus *Bifidobacterium* [27]. The taxonomic gut microbiota composition is presented in Fig. 2 [28].

The bacteria present in the gut are classified into two types: Gram-negative and Gram-positive. The types are differentiated by their stained ability by the staining technique given by Danish scholar "Dr. Hans Gram". Whether the bacteria are stained or not using Gram stain depends on cell wall structure of the bacteria (Gram-negative stains red and Gram-positive bacteria stains violet). The variations in cell wall structure shows the difference of phylogeny of Gram-negative and Gram-positive bacteria [29].

The cell wall of gram-positive bacteria is composed of a thick layer of peptidoglycan whereas cell wall of Gram-negative bacteria is composed of a thin peptidoglycan layer. Gram-negative bacteria also contain one outer membrane and lipopolysaccharides (LPS) which are responsible for number of diseases. The presence or absence of LPS is not related to pathogenicity of bacteria. Although Gram-negative bacteria have LPS in their cell wall, despite this they include both beneficial and pathogenic

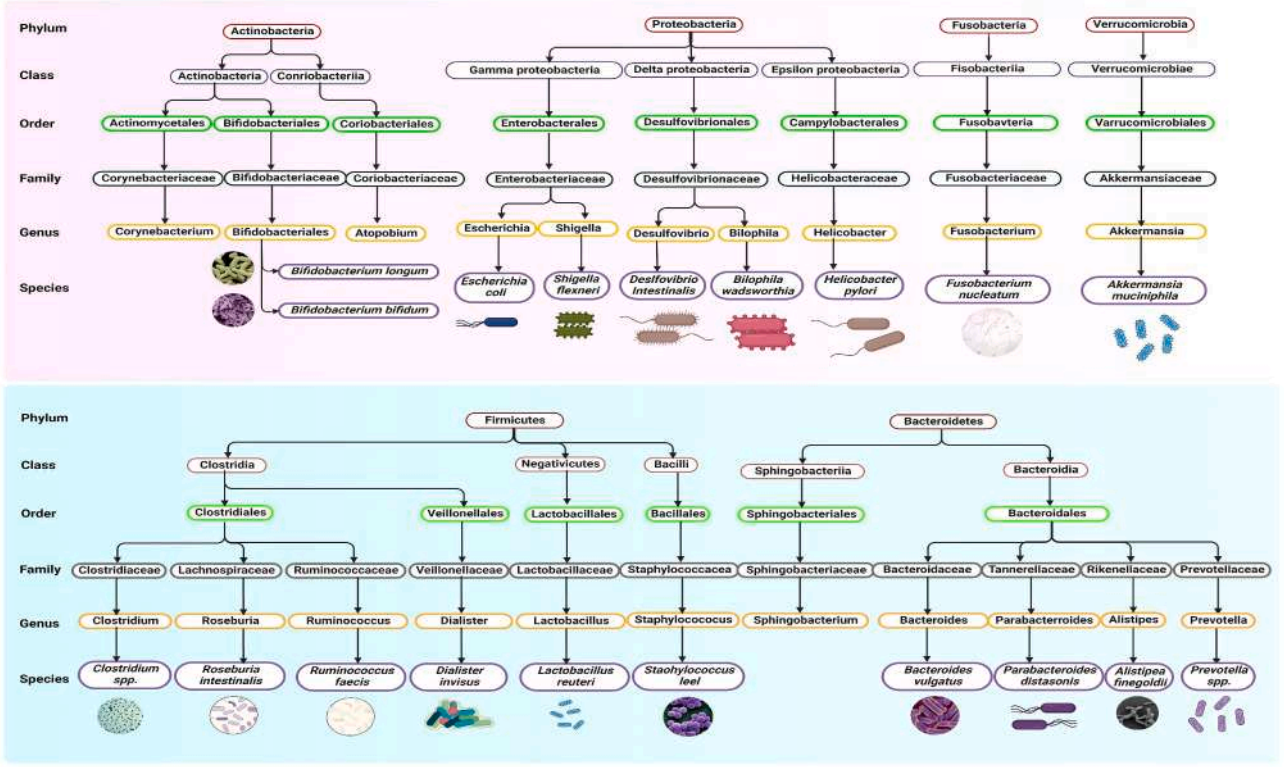

Fig. 2. Figure depicting the taxonomy of gut microbiota composition.

Table 3
Characteristic features of "good" and "bad bacteria".

Good Bacteria			
Representative bacteria	Secretions	Actions	Effect on body
Bifidobacteria	Thiamine, riboflavin, vitamin B_6, folic acid, biotin and vitamin K [217]	Vitamin synthesis, digestion and absorption, prevent infection and stimulate immunity	Maintain the overall health
Lactic acid bacteria	Lactic acid [218]		
Bad Bacteria			
Staphylococci (e.g., Staphylococcus aureus)	Alpha and gamma toxins, protease aureolysin, phenol soluble modulins, coagulases (staphylocoagulase and von Willebrand factor) [219]	Produce carcinogenic substances and toxins, intestinal putrefaction,	Trigger disease
Escherichia coli (Toxic strain)	Shiga toxin [220]		
Clostridium perfringens	Alpha (CPA), beta (CPB), epsilon (ETX) and iota (ITX) toxins [221]		
Opportunistic Bacteria			
Bacteroidetes	Antimicrobial protein 1 [BSAP-1]), catalase [222]	No trouble when healthy, but have adverse actions inside the intestines when the body is weak	–
Escherichia coli (Non-toxic strain)	Vitamin K and Vitamin B12 [223]		
Streptococci	Erythrogenic toxins (pyrogenic exotoxins) and the cytolytic toxins (streptolysins S and O) [224]		

bacteria [29]. Beneficial Gram-negative bacteria include acetic acid bacteria, *Zymomonas mobilis*, *Xanthomonas bacteria* and *Pantoea agglomerans*. On the other hand, pathogenic Gram-negative bacteria include "*Vibrio cholerae*" and "*Salmonella*" species. Moreover, Gram-positive bacteria such as lactic acid bacteria are known as beneficial bacteria whereas *Staphylococcus aureus*, *Mycobacterium tuberculosis*, etc. are the pathogenic Gram-positive bacteria. One more category of bacteria is opportunistic bacteria. In case of healthy body, these bacteria do not cause any health issues whereas in case of weak body these cause certain health issues [30]. Few of the characteristic features of these "good" and "bad" bacteria are presented in Table 3.

The human gut microbiome impacts human health in positive as well as negative ways. For instance, bacteria contain lipopolysaccharides that provide low-grade tonic stimulation of the innate immune system. However, its excessive stimulation due to bacterial dysbiosis, small intestinal bacterial overgrowth, or increased intestinal permeability produces systemic and/or central nervous system inflammation [31]. The proteins present in bacteria may cross-react with human antigens to stimulate negative responses of the adaptive immune system. The enzymes present in bacteria may produce neurotoxic metabolites such as D-lactate and ammonia [31]. It has been reported that some of the beneficial metabolites released by bacteria such as short-chain fatty acids may also exert neurotoxicity. They also produce hormones and neurotransmitters that are identical to those produced by humans. Gut bacteria directly stimulate afferent neurons of the enteric nervous system to send signals to the brain via the vagus nerve. Overall, the chemicals produced by them may have positive as well as negative impact [31].

D-lactate is produced by bacteria by carbohydrate's fermentation. This is usually produced in excess when small bowel resection allows delivery of a high carbohydrate load to the colon. Elevation of D-lactate level in plasma may cause encephalotoxicity [32–34]. The supplementation of synbiotics may limit production of D-lactic acid in the gut however, the strain should be chosen carefully. For instance, some species of *Lactobacillus* are D-lactate producers. In addition, prebiotics

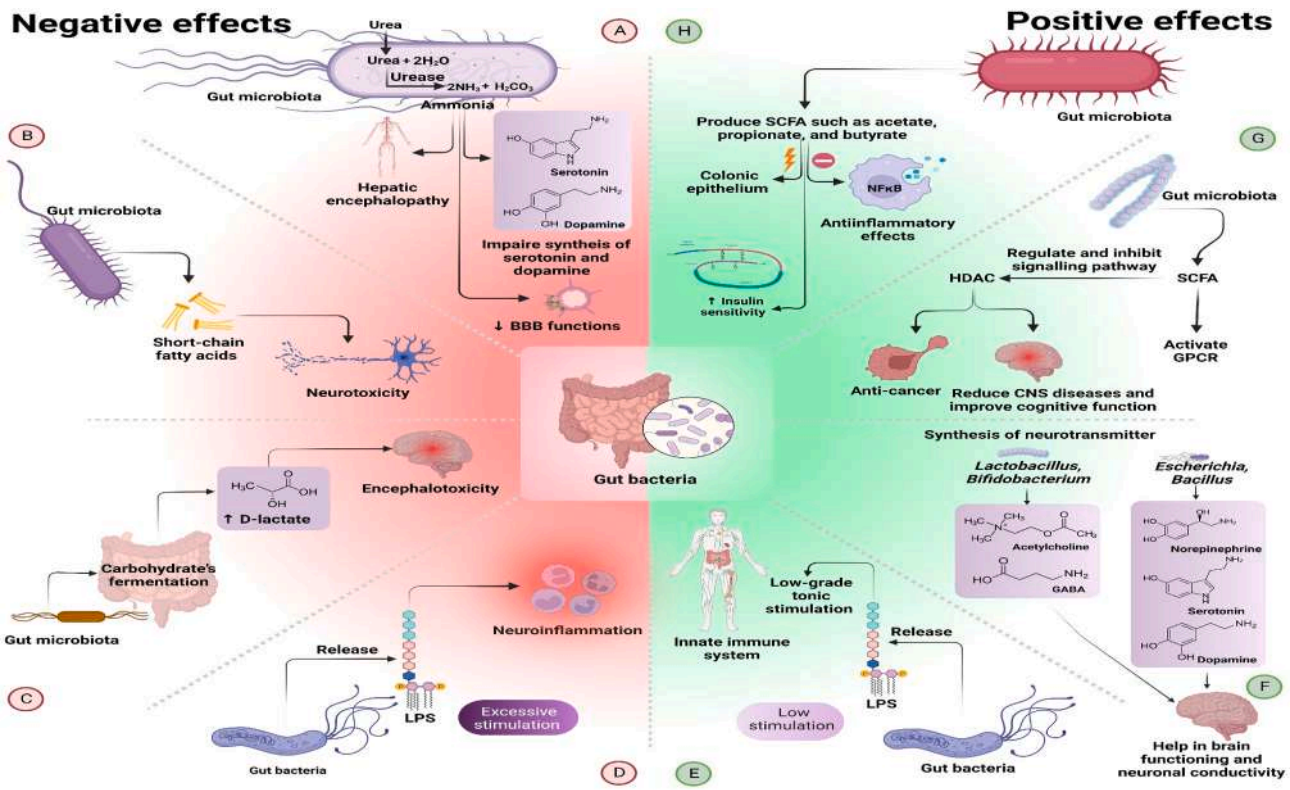

Fig. 3. Structure of chemicals released by gut microbes and their effect on body.

such as high-dose beta-glucan can increase intestinal permeability thereby leading to the secretion of D-lactate. In one of the studies, a combination of *Bifidobacterium breve* Yakult and *Lactobacillus casei* Shirota as probiotics and galacto-oligosaccharide as a prebiotic helped in reduction in colonic absorption of D-lactate by limiting the growth of D-lactate-producing bacteria and stimulating intestinal motility [35].

The gut bacteria contain enzyme ureases that produces ammonia which is a neurotoxin. This ammonia is taken up by the liver and consumed in the urea cycle. By creating portosystemic shunts, cirrhosis allows absorbed ammonia to escape hepatic metabolism, increasing blood ammonia, which contributes to the pathogenesis of hepatic encephalopathy (HE) [36,37]. Furthermore, ammonia indirectly alters function of the blood–brain barrier, impairing intracerebral synthesis of serotonin and dopamine and producing abnormal neurotransmitters such as octopamine, phenyl-ethylamine [38]. It has been reported that a combination of *Bifidobacterium longum* and fructo-oligosaccharide as synbiotics or a cocktail of four freeze-dried, non-urease-producing bacteria (*Pediacoccus pentoseceus*, *Leuconostoc mesenteroides*, *Lactobacillus paracasei* ssp. *paracasei*, and *Lactobacillus plantarum*) mixed with beta glucan, inulin, pectin, and resistant starch as prebiotics was able to alleviate the bacterial ammonia related neurotoxicity [39].

The gut bacteria ferment the indigestible carbohydrates in the normal human colon and produce short chain fatty acids (SCFA) such as acetate, propionate, and butyrate [40]. Butyric acid is reported to supply 70% of energy requirements of the colonic epithelium [41] and has direct anti-inflammatory effects. It also inhibits activation of nuclear factor kappa-B (NFkB) [42]. Similarly, propionic acid also inhibits NFkB and reported to improve insulin sensitivity by activating peroxisome proliferator-activated receptor gamma [43]. SCFA regulate molecular signalling of neurons by causing histone deacetylation (HDAC) and activating G-protein-coupled receptors (GPCRs) [44]. Acetylation and deacetylation of the histone proteins around which DNA coils cause epigenetic regulation of gene expression. An imbalance in the direction of excessive HDAC is reported among patients suffering from Parkinson's disease [45], depressions, and schizophrenia [46]. Inhibition of HDAC has beneficial effects in cancer and a number of animal models of CNS disease, including brain trauma, dementia, and autoimmune encephalitis [47,48]. The SCFA produced by gut bacteria are also histone deacetylase inhibitors and cause enhancement of cognitive function [49].

Gut bacteria has the ability to synthesize and respond to hormones and neurotransmitters. For example, acetylcholine and gamma-amino butyrate (GABA) are produced by *Lactobacillus* species. GABA is also produced by *Bifidobacterium* species. Norepinephrine, serotonin and dopamine are produced by *Escherichia* species. Serotonin is also produced by *Streptococcus* and *Enterococcus* species and norepinephrine and dopamine are also produced by *Bacillus* species [50]. These neurotransmitters play significant role in neuronal conductivity as well as proper functioning of nervous system in human. The structure of these chemicals released by gut microbes and their role is shown in Fig. 3.

2.1.1. Change in composition of gut microbiota with age

The foetus lives in an entirely germfree environment in utero in the mother. In uterus, the foetus lives in an entirely germfree environment and after birth, bacteria quickly colonises it. After birth, *Enterococcus, Escherichia coli, Staphylococcus*, *Lactobacillus, and Clostridium* are present in the faecal matter of almost all new-borns, having the total bacterial counts up to 10^{11} per gram [51].

In breast-fed infants, *Bifidobacterium* normally starts to appear about 3 days after birth, and the bacterial strains which were already present start decreasing. Between day 4 and day 7, *Bifidobacterium* becomes predominant, with counts of 10^{10} to 10^{11} per gram. In contrast, compared to *Bifidobacterium* there is a 1% reduction in the bacterial counts of *Clostridium*, *Escherichia coli, Staphylococcus, Streptococcus, and Bacteroides*. On 7th day, the gut microbiota composition becomes stable [52].

As the weaning period starts, the gut microbiota starts, Gram-negative rod flora becomes predominant in adults with a total count

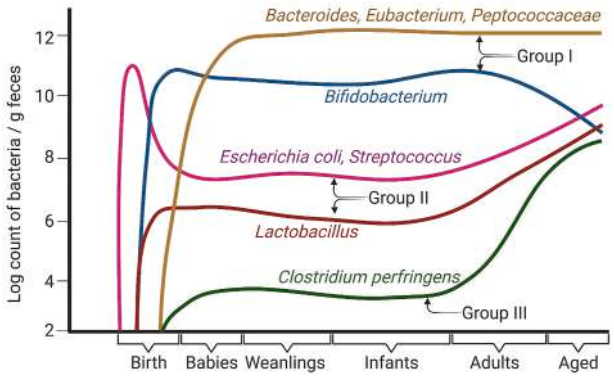

Fig. 4. Graph indicating change in microbial count among humans with respect to aging.

of about 10^{11} per gram. At this stage, *Bifidobacterium* decreases to about 10% of the total flora whereas *Bacteroides, Streptococcus* and often *Clostridium count starts increasing* [53].

The gut microflora again shows some variations during the transition from middle age to old age. The *Bifidobacterium start reducing and* in some cases become totally untraceable during old age. But, the count of *Clostridium perfringens* remarkably increases. Moreover, the counts of *Lactobacillus* and *Enterococcus* also increase. This change in bacterial count is due to the effect that aging of physiologic function in the host has on the gut flora. This in turn may itself further increase the process of aging (Fig. 4) [51].

3. Role of synbiotics in management of various diseases

3.1. Gut microbiota and DM

DM is attributed to reduced insulin secretion which could be attributed to β-cell damage or insulin resistance (IR) [54,55]. In case of DM, the gut dysbiosis is reported to result in reduction of useful butyrate producing Gram-positive bacteria and overgrowth of Gram-negative bacteria containing lipopolysaccharides (LPSs) in their cell membrane as well as in opportunistic pathogens. A significant alteration in both α and β diversity of microbiota is observed [56]. As a result of dysbiosis and increased LPSs concentration, chronic inflammation gets induced, which in turn, leads to a compromise in the gut mucosal barrier integrity, affecting the tight junctions, leading to increase in the intestinal permeability. Bacteria, their metabolites as well as LPSs get translocated from the gut epithelium and enter into the blood stream [57]. All these lead to IR, endotoxemia, inflammation, obesity and DM. The mechanism linking inflammation and IR is based on the activation of IkB (nuclear factor of kappa light polypeptide gene enhancer) kinase complex. IkB kinase inhibits nuclear factor kappa beta, which is responsible for inflammation. This leads to IR by reducing the tyrosine phosphorylation of insulin receptor substrate proteins [58].

There is sufficient evidence to suggest that gut dysbiosis is one the factors involved in the development of DM. A study conducted by Sato et al. (2014) on diabetic Japanese patients correlated the gut microbiota disturbances with IR. Concentration of *Clostridium coccoides* group and *Prevotella* (obligate anaerobes) was found be less while the concentration of *Lactobacillus* (facultative anaerobes) was observed to be increased in the faecal samples of diabetic patients as compared to those of control subjects. The blood samples of diabetic patients (28%) also showed a significant number of gut bacteria (mostly Gram-positive) compared to that in control subjects (4%). This suggests that the bacteria present in the gut had been translocated into bloodstream due to compromise in tight junctions, ultimately causing endotoxemia and inflammation [59].

When synbiotics are administered, the prebiotic component increases the viability of probiotics and serves as a nutrition source for them. Once the probiotic component reaches intestine, it multiplies and the number of Gram-positive bacteria increases leading to a reduction of Gram-negative bacteria and LPSs. Moreover, synbiotics improve the epithelial barrier function and decrease the adherence of opportunistic pathogens in the intestine. This further reduces gut dysbiosis, intestinal permeability, endotoxemia, and IR [60]. In some studies, the effect of synbiotics on DM has been associated with an enhancement of colonic SCFAs levels, particularly butyrate [61]. SCFAs are known to be quite important for the growth of *Bifidobacteria* and *Lactobacilli* which are anticipated to have useful effects in diabetics [62].

Synbiotics are also reported to exert an effect on increased protein expression involved in the insulin-signaling pathway, including insulin receptors [insulin receptor- β (IR-β), insulin receptor substrate-1 (IRS-1) and protein kinase-β (Akt)]. This results in an enhanced uptake of glucose and a reduction in blood glucose level (BGL) [63]. Synbiotics are known to decrease the oxidative stress and increase the antioxidants levels of superoxide dismutase (SOD) and glutathione peroxidase (GPx) [64]. They also suppress inflammatory cytokines and increase interleukin-10 (IL-10) production. IL-10 reduces the levels of pro-inflammatory cytokines including high-sensitivity C-reactive protein (hs-CRP), tumor necrosis factor-alpha (TNF-α) and T helper (Th1) cytokines and stops β-cell destruction [65,66]. Glucagon-like peptide 1 (GLP-1) prevents postprandial hyperglycemia by improving release of insulin from pancreas [67]. Several studies [68] have also indicated towards beneficial activities of probiotics on insulin sensitivity, which were associated with enhanced GLP-1 levels.

3.1.1. Role of synbiotics: Evidence from preclinical studies and clinical studies

A number of preclinical studies and clinical trials have reported the beneficial effects of synbiotics in management of hyperglycaemia by reducing gut dysbiosis.

Morshedi et al. (2020) carried research wherein the effect of synbiotics (inulin and *L. plantarum)* was investigated in type 2 DM (T2DM) rats. Synbiotics were found to be more effective in reducing gut dysbiosis as well as oxidative stress in comparison to both prebiotic and probiotic given individually. Although inulin and *L. plantarum,* both were able to reduce malondialdehyde (MDA) concentration and increase SOD, GPx and total antioxidant capacity (TAC), the result was found to be more pronounced in case of combination. Furthermore, the largest ratio of *Bacteroides/Firmicutes* (14.4%) and *Clostridium/Firmicutes* (12.2%) found in the hyperglycaemic rats was overturned more prominently by intake of synbiotics in comparison to that by pro and prebiotic intake alone. Another notable observation in this metagenomics study which was observed only in the synbiotic group, was the dramatic enhancement in the population of *L. plantarum*, which are the Gram-positive lactic acid bacteria. Other significant outcomes of the study included an enhancement in the concentration of serotonin as well as brain derived neurotropic factor/tropomyosin receptor kinase B/cyclic AMP response element binding protein (BDNF/TrkB/CREB). BDNF and serotonin play an essential role in enhancing as well as maintaining neuropsychological functions and averting nerve injury [69].

In a recent study conducted by Miller et al. (2021), it was observed that a specially developed synbiotics yogurt was able to reduce BGL to an extent much higher than that by the commercially available milk as well as yogurt. In fact, the control yogurt was found to enhance the population of harmful bacteria such as those from families Enterobacteriaceae and Proteobacteria and worsen the development of DM. The islet morphology of pancreas was found to be protected by synbiotics yogurt as compared to that by milk control while a negative effect was observed in case of the control yogurt [70]. In another study, conducted on diabetic rats, the individual administration of insulin, pioglitazone and synbiotics (*B. bifidum* and FOS) was found to reduce BGL, improve lipid profile, reduce triglyceride (TG), total cholesterol (TC) and low density lipoprotein-cholesterol (LDL-C) and reduce the oxidative stress. However, the effect was more significant when all the three components

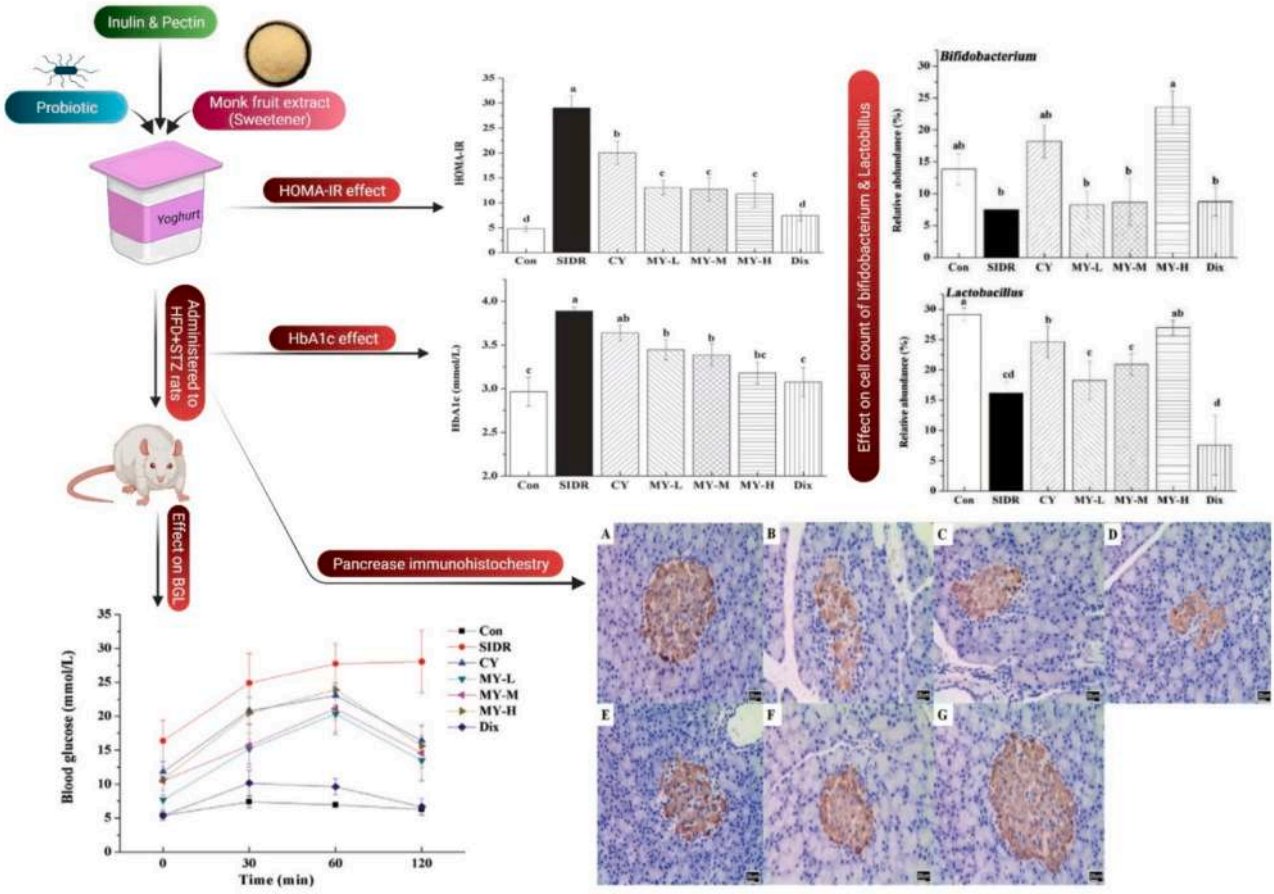

Fig. 5. Effect of synbiotics on diabetic rats. Reprinted with permission from Ref. [73]. Copyright 2020, Elsevier.

were given in combination [71].

In another study, consumption of synbiotics product of yacon extract, soyabean and fermented *Enterococcus faecium* and *L. helveticus* significantly decreased the level of TGs and improved the level of high density lipoprotein-cholesterol (HDL-C) [72]. Ban et al. (2020), formulated a synbiotics yogurt by using monk fruit extract as a sweetener and studied its effects in T2DM rats. The rats exhibited more pronounced regulation of BGL and a substantial reduction in IR and glycated haemoglobin (HbA1c) in comparison to rats administered with sucrose sweetened yogurt. The synbiotics also caused a significant enhancement in SCFA levels and improved the status of gut dysbiosis. Administration of synbiotics was also reported to reduce the kidney and liver injury. The outcomes of immunohistochemistry examination revealed that the synbiotics yogurt repressed β-cell damage and loss. Furthermore, it restored the islets of Langerhans to a greater extent as compared with that by the control yogurt [73]. This study has been represented in Fig. 5.

In a randomized clinical trial conducted on 62 diabetic patients, the administration of synbiotics comprising of inulin and *L. sporogenes* led to a remarkable reduction of IR and hs-CRP [74]. Kooshki et al. (2015) reported that administration of synbiotics tablets in diabetic patients led to a remarkable reduction in inflammatory biomarkers (hs-CRP and IL-6) as compared to that by placebo. The DB, placebo controlled trial was conducted on 44 patients for 8 weeks [75]. Another randomized, DB clinical study was carried on 60 pregnant females with gestational DM who were administered capsules containing synbiotics (*L. casei, L. acidophilus, B. bifidum* and inulin) for 6 weeks. The results were compared with those of placebo. It was seen that administration of synbiotics remarkably decreased MDA, hs-CRP, and increased TAC and GSH levels [76,77]. Similar results were noted in terms of reduction in MDA and hs-CRP in another randomized, DB, clinical trial conducted on 60 diabetic people by administration of synbiotics containing *L. acidophilus* and *B. bifidum* along with inulin for 12 weeks [65]. Shakeri et al. reported that the administration of synbiotics bread loaded with *L. supergenes* and inulin for 8 weeks to 26 diabetic patients, reduced serum triacylglycerol (TAG) and very LDL-C (VLDL-C), and increased serum HDL-C levels compared to the probiotic and control groups [78]. Similarly, in another randomized, DB, clinical trial, synbiotic bread proved to be more effective than probiotics alone, in reducing IR in a *homeostatic model of assessment for insulin resistance (*HOMA-IR) and in improvement of pancreatic β-cell function [79]. Further, treatment with synbiotics capsules composed of *L. casei*, *L. acidophilus*, *B. bifidum and inulin for 12 weeks, decreased BGL, HOMA for* β*-cell function (HOMA-*β*) and increased the* quantitative insulin sensitivity check index in obese diabetic patients [80]. Synbiotics supplementation in obese T2DM patients for 24 weeks was found to be effective in increasing the population of beneficial species of bacteria (Bifidobacterium and total *Lactobacilli*) leading to reduction in the gut dysbiosis [81]. In another randomized, DB, clinical trial enrolling 50 type 1 DM (T1DM) patients, it was observed that synbiotics containing *L. sporogenes* GBI-30 as probiotic and maltodextrin and FOS as prebiotic, supplementation for 12 weeks decreased HbA1c, hs-CRP and BGL and increased TAC levels [82].

3.2. Gut microbiota and polycystic ovarian disorder (PCOS)

PCOS is a polygenic, hormonal disorder affecting females during their reproductive age. PCOS is linked with lack of ovulation and infertility related to metabolic/hormonal imbalances including hyperandrogenism, IR, systemic inflammation and hypercholesterolemia [83,84]. Gut microbiota controls numerous physiologic functions which are

pathologically altered in PCOS i.e., systemic inflammation, glucose metabolism, and energy homeostasis. Dysbiosis has been reported as a major contributing factor in PCOS pathogenesis [85]. Many clinical and preclinical studies in rodent models indicate a significant relation between PCOS and gut microbiome [86,87]. The theory of ''Dysbiosis of Gut Microbiota'', states that the immune system of host can be stimulated by the gut dysbiosis, activating a chronic inflammatory response which damages functioning of insulin receptors resulting in IR. The resulting increase in insulin levels interferes with follicular growth, while stimulating excessive androgen synthesis by the thecal cells of ovary [88]. In addition, changes in the gut microbial composition increase the testosterone production [89]. In a recent study, even the dysbiosis of saliva was found to be associated with PCOS [90]. Synbiotics are anticipated to improve the composition of gut microflora, decrease leaky gut, inflammation, and improve insulin sensitivity [4]. They are, therefore, recommended as supplements to prevent or manage PCOS [83,84].

Administration of synbiotics consisting of inulin, fructooligosaccharide and *L. acidophilus* was found to reverse the experimentally induced PCOS in female Wistar rats [91].

There is some clinical data available that suggest the role of synbiotics in treatment of PCOS. In one of the randomized, DB, placebo controlled clinical trial, 23 PCOS women were given synbiotics comprising of pomegranate juice as prebiotic along with probiotic bacteria. The group treated with synbiotics showed a remarkable reduction in testosterone, IR, and body mass index (BMI) and an increase in insulin sensitivity [92]. In another study, synbiotics capsules (*L. acidophilus, L. bulgaricus, L. casei, B. breve,* and *Streptococcus thermophilus* + prebiotic inulin) were administered to 44 PCOS women for 12 weeks. The synbiotics decreased the levels of apelin, which is an endogenous ligand for the G-protein-coupled APJ receptors and is generally increased in PCOS women [93]. Samimi et al. (2018) conducted a randomized, DB study in 60 females suffering from PCOS. Out of these, 30 women were supplemented with synbiotics capsules. In comparison to placebo, the synbiotics treatment exhibited a remarkable reduction in serum insulin, HOMA-IR, serum TGs, and VLDL-C [94]. Synbiotics supplementation in 30 PCOS women for 12 weeks was also reported to increase serum sex hormone-binding globulin (SHBG), plasma nitric oxide (NO) and reduced hs-CRP levels in comparison to placebo [95]. Moreover, consumption of synbiotics pomegranate juice for 8 weeks improved oxidative and inflammatory markers in PCOS women in comparison to those by placebo [96].

3.3. Gut microbiota and NDs

NDs, such as Alzheimer's Disease (AD), Parkinson's Disease (PD), and Lewy body disease refer to a diverse group of disorders which lead to peripheral or/and central nervous system (CNS) deterioration. Among all the NDs, the prevalence rates of AD and PD are very high. It is estimated that about 8% and 1% of the people, globally, are effected by AD and PD respectively [97]. The microbiota-gut-brain axis, represents the interactions between the CNS and the enteric nervous system (ENS) within the GIT via a bi-directional communication system composed of immune, neural, and endocrine pathways [98]. Gut has been reported to be involved in the synthesis of a number of bioactive peptides and hormones [99]. The immune cells, highly chemo-sensitive primary afferent neurons, and enteroendocrine cells, release about 30 different hormones that are responsible for accomplishment of gut to brain signalling in the gut. The immune pathways, including cytokine signalling activated by peptidoglycan or bacterial LPSs, also serve as a communication link to the brain. Any alterations in the integrity of gut barrier may result in translocation of these bacterial products in the systemic circulation with downstream microglial activation and neuroinflammation [100]. Moreover, the proinflammatory cytokines activate and trigger the hypothalamic pituitary axis (HPA) which, in turn, leads to release of cortisol which contributes to neurodegeneration. Various research studies point towards gut dysbiosis as a causative factor in NDs [101]. Some of these involve microbiota-gut-brain-axis, while some other pathways are presently under investigation. Further studies need to be undertaken to explore various mechanisms through which the gut bacteria influence this axis. Bacteria are known to synthesize neurotransmitters, stimulate serotonin synthesis by gut epithelial cells, modify epigenetic regulation with by-products of fermentation, synthesize bioactive constituents, and release metabolites which can go into the systemic circulation and cross the blood brain barrier (BBB) [102]. Dysbiosis of the gut results in dysfunction of the gut brain axis signalling, which increases inflammation, oxidative stress as well as metabolism and immune function's disbalance [103].

In case of AD, amyloid plaque and tau tangles get formed in the brain. Dysbiosis of the gut is known to contribute to the etiopathology of AD by enhancing formation of amyloid-β, increasing oxidative stress, neuroinflammation, and IR. Gut dysbiosis results in the reduction of SCFAs and increase in amyloids and trimethylamine N-oxide (TMAO), which increases intestinal BBB permeability, and stimulates peripheral immune responses [104].

PD involves the build-up of α-synuclein protein which leads to degeneration of dopaminergic neurons in the pars compacta of the substantia nigra (SN) slowly and progressively. There is also convincing evidence that the accumulation of α -synuclein initiates in the gut, years before affecting the CNS. This is further aggravated by dysbiosis, where an alteration in the diversity of intestinal bacteria, possibly results in local inflammatory response with erratic permeability of the intestinal epithelium, permitting some microbial products to reach the circulation, which then cross the BBB to reach brain [105].

In some studies, both, the patients suffering from NDs and the aged people, showed decreased gut bacterial biodiversity [106,107]. Studies correlating the effects of synbiotics in NDs such as AD have also emerged recently [103].

A novel synbiotics composition consisting of polyphenol extract from a polyherbal formulation, Triphala as prebiotic and *L. plantarum*, *L. fermentum* and *B. longum* as probiotic were tried in *Drosophila melanogaster* model of AD. Synbiotics was found to result in their increased viability and motility and downregulated deposition of amyloid β and acetylcholinesterase activity. Such extreme actions were found to be achieved due to the synbiotics combinatorial activity on GBA signalling pathways together with immune signalling, metabolic stability, mitochondrial and oxidative stress, perhaps through pathways involving peroxisome proliferator-activated receptor gamma (PPARγ) [108]. In a study using the *ex vivo* model of the human GIT, use of synbiotics comprising of combination of grape-derived polyphenolic precursors as prebiotics and 3 strains of bacteria (*L. salivarius, L. plantarum, B. infantis)* using a multivariate regression algorithm (MARS) led to a synergistic effect. An improvement in the brain bioavailable metabolites including kamferin, quercetin, and 4-hydroxyphenylacetic acid could be observed. It was also observed that both the metabolites i.e., 3-hydroxybenzoic acid and 3-(3′-hydroxyphenyl) propionic acid extracted from grape seed were able to cross the BBB and prevent the amyloid plaques and tau fibril accumulation. When the polyphenolic pool diversity was increased by addition of resveratrol and grape extract, higher protection towards intellectual decline and amyloid accumulation was attained in AD mouse than with any of the components alone [109].

Administration of synbiotic consisting of xylo-oligosaccharide (XOS) as prebiotic and *L. paracasei* as probiotic, in obese insulin resistant rats, reduced apoptosis and oxidative stress and consequently re-established cognition through gut-brain axis, resulting in amended and improved mitochondrial function of brain, hippocampal plasticity, insulin sensitivity and reduced microglial stimulation [110]. Another study aimed at evaluating the outcome of a nutritional synbiotic supplement on memory assessment and anxiety response in heat-stressed broiler chickens. Synbiotics containing a combination of probiotics (*Pediococcus acidilactici*, *Enterococcus faecium*, *L. reuteri*, and *B. animalis*) and a prebiotic (FOS) were administered to broiler chickens at 3 doses (0, 0.5, and 1.0

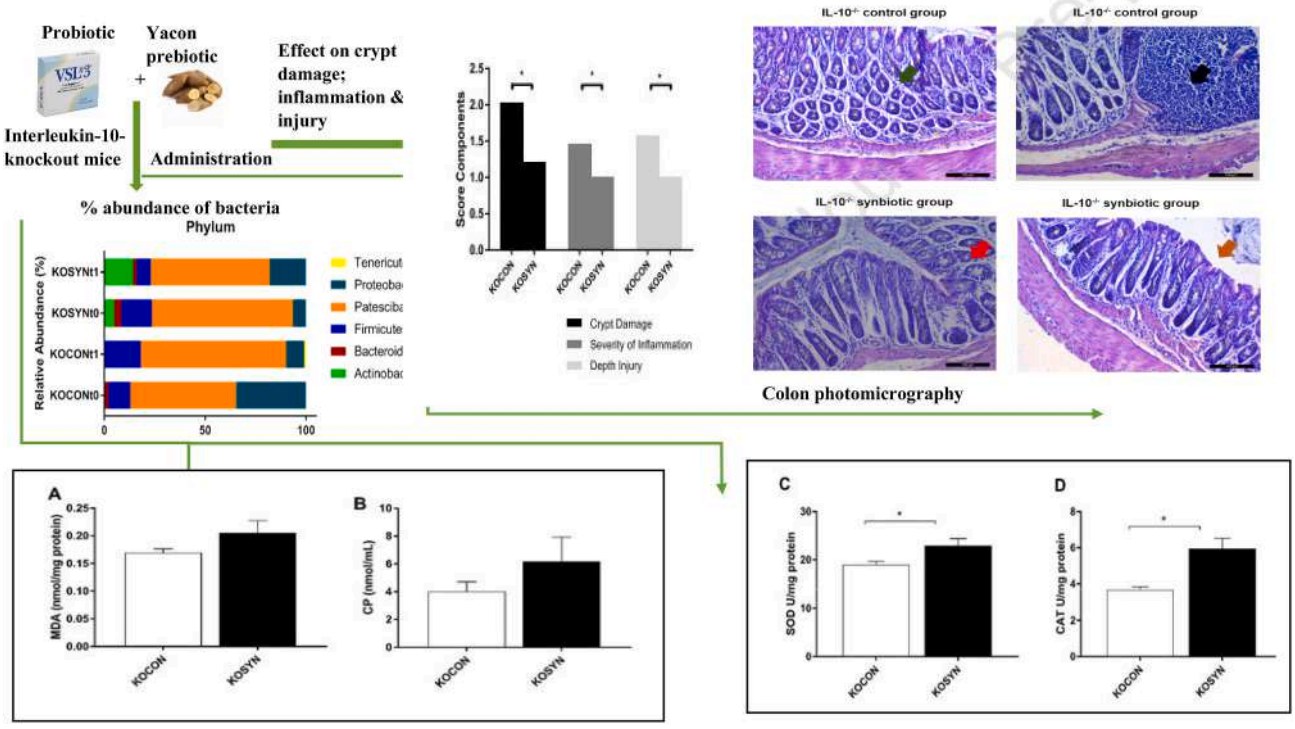

Fig. 6. Effect of synbiotics on carcinogenic mice. Reprinted with permission from Ref. [131] Copyright 2020, Elsevier.

g/kg). The results signified that the synbiotics decreased heat-stress responses and associated emotional disorders which were attributed to enhanced stimulation of the serotonergic system through the microbiota–gut–brain axis [111]. In an uncontrolled clinical trial, kefir synbiotic milk was administered to patients suffering from AD. It was observed that the administration of synbiotic kefir led to recovery of the cerebral deficits, which appears to be associated with three significant features of the AD i.e., oxidative stress, systemic inflammation and blood cell damage [112].

3.4. Gut microbiota and CVDs

The CVDs which involve heart and blood vessels include disorders like hyperlipidemia, atherosclerosis, myocardial infarction (MI), stroke, and cardiomyopathy. Gut microbiota are known to exert numerous effects on the cardiovascular system of the host [113]. Certain metabolites produced by microbiota like trimethylamine/TMAO, SCFA, and bile acids are involved in interaction with the cardiovascular system of the host. Increased TMAO results in foam cell production and therefore imposes a risk for atherosclerosis and other CVDs. Certain metabolism-independent processes are also thought to be involved in the pathogenesis of CVDs. For example, bowel wall edema, cardiac failure-linked splanchnic circulation congestion, and disrupted intestinal membrane can cause microbial translocation, along with the appearance of products such as LPSs in the blood, leading to inflammation. These are believed to be contributing factors for further development of cardiac failure and atherosclerosis [114].

In a clinical study in patients with CVDs risk burden, alteration in the profile of microflora was found to be linked to a much higher risk [115]. A decreased *Bacteroidetes*: *Firmicutes* ratio is linked to weight gain, and vice versa for weight loss [116]. Similarly, reduced diversity of bacterial population and reduced ratio of *Bacteroidetes* to *Firmicutes* was observed in hypertensive animals. In another study, the patients suffering from atherosclerosis were reported to have an increased proportion of *Collinsella in comparison to* normal group who were supplemented with *Eubacterium and Roseburia*. Abundance of *Akkermansia muciniphila* in obese mice has been connected with inflammation and lipid metabolism in adipose tissue. The study showed numerous links between metabolic syndrome and the gut microbiota [117]. Administration of synbiotics has proven to be effective in managing CVDs via reduction of gut dysbiosis. Synbiotics are proposed to reduce the level of TC, and may reduce CVD risk, by enhancing bile salt production and bile acid deconjugation. In addition, synbiotics also exhibit anti-platelet, anti-oxidative, and anti-inflammatory properties [118].

Sarfraz et al. (2019) studied the effect of synbiotic yoghurt containing iso-maltooligosaccharide and FOS as prebiotics, and L. acidophilus as probiotic in hyperlipidemic rabbits. A substantial reduction in the levels of TGs, TC, LDL and VLDL-C was observed, while the levels of HDL were found to be increased [119]. Similar effects were noted when synbiotics containing inulin, mannitol, and FOS along with *L. acidophilus* were administered in hypercholesteraemic pigs. Membrane permeability and fluidity also improved in synbiotic group resulting in reduction of erythrocyte deformation [120]. The NO function as well as release was recovered, resulting in normalizing the systolic blood pressure in rats after administration of Prodefen® Plus (marketed synbiotic preparation) for 4 weeks [121]. In another study, synbiotic drink containing kefir milk as source of probiotics and jicama concentrate as prebiotics source was administered to rats for 4 weeks. The synbiotic group showed significant reduction in MDA level and increased the SOD activity [122]. Tunapong and the co-authors determined the effect of synbiotics administration containing XOS as prebiotics and *L. paracasei* as probiotic in rats for 12 weeks. Synbiotics treatment showed a remarkable improvement of lipid profiles. The parameters such as blood pressure, left ventricular dysfunction and heart rate variability showed a significant improvement [123].

In one of the randomized, DB clinical trials, the co-supplementation effects of synbiotics i.e. *L. rhamnosus* and inulin were checked in patients suffering from coronary artery diseases. Administration of synbiotics for 8 weeks had significant useful effects on anxiety, depression, and inflammatory biomarkers. The hs-CRP, LPS and TNF-alpha were found to be reduced in the synbiotic group. The effect was more pronounced when inulin was added as prebiotic as compared to that when inulin or

probiotic were given alone [124].

3.5. Gut microbiota and colorectal cancer

Etiopathology of colorectal cancer involves an interplay among the normal cells, tumoral cells, and gut microflora. Dysbiosis has been widely reported as a contributing factor in colorectal cancer. Certain species of gut bacteria are involved in the development of colorectal cancer. These include *Helicobacter pylori*, *S. bovis*, *Bacteroides fragilis*, *Fusobacterium* and *Escherichia coli* [125]. Dysbiosis of gut results in imbalance in microbial homeostasis, which, in turn, leads to carcinogenesis via different pathways, such as increasing DNA damage, driving inflammatory response, and/or endorsing cell proliferation. Nature and extent of differences in the gut microflora species during tumorigenesis have been suggested as biomarkers and diagnostic tools for colorectal cancer [126]. Oxidative stress and inflammation in the intestinal epithelium are reported to be related to colon cancer and the interruption of the colonic barrier integrity could enhance the colonocytes exposure to contaminants from the intestinal environment. These further increase inflammation and reactive oxygen species (ROS) generation [127]. Use of synbiotics in treatment of colorectal cancer is well reported [7]. They are reported to be involved in modification of metabolic activities of intestinal microflora, carcinogen binding, colon physicochemical conditions, fatty acid production, anti-mutagenic synthesis, and host immune response promotion [128].

Recent research has recommended that prebiotics also hold defensive effect against colon cancer, primarily ascribed to the synthesis of SCFAs upon their fermentation by gut microflora, and modification of gene-expressions in cancer cells. Synbiotics have been found to show a synergistic effect in reducing development of colon cancer compared to when both components were used alone [128–130].

The effect of the synbiotic containing VSL#3® and yacon-based concentrate and FOS along with inulin was studied in the mice model of cancer. The mice were categorised into 2 groups i.e., control and treatment group. Administration of synbiotics resulted in the protection of intestinal cell structure, enhanced antioxidant enzyme levels (SOD; CAT; MDA; Carbonyl proteins) and increased the SCFA content. Further, the control group showed 1.2-fold reduction of phylum *Patescibacteria* but the synbiotic group showed a 1.3-fold increase in this phylum. Similarly, the synbiotic group showed a 1.6-fold reduction and 2.1-fold increase in phylum *Firmicutes* as compared to that in the control group. Similarly, enhancement of *Proteobacteria* (2.7-fold) and its reduction (3.8-fold) was noted in synbiotic and control group respectively. The synbiotics group successfully improved the histopathological changes, such as reduction of crypts damage. Collectively, these factors alleviated the indicators of colitis and enhanced intestinal integrity, signifying the synbiotics as beneficial nutraceuticals for management of intestinal carcinogenesis (Fig. 6.) [131].

Gallaher et al. (1999) studied the synergistic effect of synbiotics (*Bifidobacteria*, Oligofructose, soybean oligosachcharide, and wheat bran oligosachcharide) on male Wistar rats. Synbiotics combination was found to significantly reduce the aberrant crypt foci (ACF) as well as colonic mucosa proliferation whereas no effect was seen when prebiotic or probiotic were used alone indicating the synergistic effect of the combination [24].

The anticancer effect of synbiotic combination (L. gasseri and extract of triccupidata leaf) was checked in mouse model of colitis associated colorectal cancer, induced by azoxymethane. A ten-week treatment of Synbiotics comprising L. gasseri and Cudrania tricuspidate was administered. It was observed that the synbiotic combination reduced the colonic mucosa damage and also decreased the colonic tumors. Also, it was revealed that the pro-inflammatory cytokines and enzymatic inflammation in mucus layers and tight junctions were upregulated by the use of synbiotics. A reduction in Staphylococcus count and an increase in Lactobacillus, Bifidobacterium and Akkermansia counts was observed, thus enhancing the protectiveness of gut microbiota. Moreover, the level SCFAs were also found to be increased [132].

A study was done on the preventive actions of Djulis (prebiotic dietary fiber) for its anticancer property in rats. After analysing for 10 weeks of administration of synbiotics as well as Djulis alone, it was observed that the number of total ACF, mucin producing ACF and Mucin-depleted foci were reduced significantly by both prebiotic as well as synbiotic treatment. Also, synbiotic treatment decreased the quantity of proliferating cell nuclear antigen and increased the quantity of apoptosis related proteins. This suggested that the synbiotics have exhibited the most prominent effect on inhibition of colon carcinogenesis via regulation of inflammatory and apoptotic pathways [133].

Effect of XOS fermented soymilk containing *Weissella cibaria* (FSMXW) was determined against colon cancer cell proliferation. This treatment was then compared with the effect of soymilk inoculated with *L. rhamnosus*. The results showed that the presence of XOS and *Weissella cibaria* enhanced the functional and nutritional properties of soymilk through the fermentation process. It also increased viscosity and acidification rate. It also reduced Caco-2 proliferation by decreasing the transcription of TLR4, MDA, and NF-κβ. On the other hand, such effect was not seen with soymilk inoculated with *L. rhamnosus* indicating that a specific synergistic combination of probiotics and prebiotics is mandatory to achieve the synergistic effects of the two [134].

In another study, anticancer and antioxidant effects of purified antioxidant peptides from raw peptide extract of yoghurt containing pineapple peel powder with two β-casein-derived peptides, 193YQEPVLGPVRGPFPIIV209 and 69SLPQNIPPLTQTPVVVPPF87 (designated as P17 and P19, respectively), were determined on HT-29 cell line. P17 exhibited increased antioxidant action against 2,2′-azinobis (3-ethyl benzothiazoline-6-sulphonic acid) radicals, with an IC50 value of 29.88 μg/mL, in comparison to P19 (IC50 1.44 mg/mL). Moreover, HT-29 proliferation was suppressed (41.49% and 38.55%, respectively, by P17 and P19 at 3 mg/mL) through induction of apoptosis and cell cycle arrest in G2/M-phase [127].

The synbiotic treatment (*L. casei* and *B. breve* + 4^{G}-β-Galactosyl-sucrose) in cancer induced mouse was found to reduce mortality by suppression of the expression of COX-2, STAT-3, IL-6, and TNF-α gene transcripts in colonic epithelium. However, no effect was seen on inflammation and tumorigenesis in prebiotic as well as probiotic alone treated group [135]. Another study was done on male Wistar rats to demonstrate the effect of synbiotics (Yacon or *Smallanthus sonchifolius* + *L. casei*) for treating colon carcinogenesis induced by 1,2 dimethlhydrazine (DMH). The synbiotics treated group showed a decrease in the number and multiplicity of ACF and invasive adenocarcinomas. Tumor multiplicity and cell proliferation of colonic crypts and tumors were also found to be reduced in the synbiotics group [136]. The synergistic effect of lycopene and synbiotics was studied in early biomarkers of rat colon cancer. The rats were administered lycopene or synbiotic (*B. lactis* + oligofructose/insulin) or their combination. It was observed that the combination treatment as well as treatment using all the components individually increased apoptosis and decreased the Proliferating Cell Nuclear, P53 labelling indices and ACF [137]. Treatment using combination of 6 probiotics (*L. acidophilus, S. thermophilus, Bifidobacterium* sp., *L. casei, L. delbrueckii* subsp *Bulgaricus and B. longum) and* chicory FOS as prebiotics was reported wherein 100% survival was reported, whereas the group which received only carcinogen (1,2-dimethylydrazine) had 70% survival rate. The synbiotic treated group showed remarkable reduction in inflammation, ACF dysplasia and colitis-like lesions [138]. and the incidence and multiplicity of colonic cancer in comparison to control group. The addition of prebiotic altered the fermentation events [SCFA, pH] whereas the addition of *B. lactis* into the diet did not show any effect on the fermentation parameters. This proved the superiority of synbiotic therapy over the individual components [139].

In another study, rats fed with diet containing moderate-resistant starch and *B. lactis* showed higher acute apoptotic response to a genotoxic carcinogen in the colon compared to rats administered with a diet without *B. lactis*. SCFA levels and count of *Bifidobacteria* and *Lactobacilli*

Table 4
Different approaches for colon targeted drug delivery.

Approaches	Description	Advantages	Limitations	References
pH-dependent systems	The formulation is filled with enteric coated polymers and drug is released at alkaline pH	In this system polymers are able to withstand in stomach at lower pH and release the drug at high pH in colon	- Less reliability - The pH of GIT gets completely disturbed and become alkaline throughout GIT. In that case the pH-based polymer is unable to restrict the drug in upper GIT	[225]
Microbially triggered systems	Covalent bonding is formed between drug and carrier	Drug is protected in acidic environment by polymers which release the drug in intestine	The prodrug technique is not adaptable because the formulation of drug is dependent on its functional moiety and availability of specific enzymes in the GIT to breakdown the prodrug and release the active drug	[226–229]
• Prodrugs	It is triggered by the bond cleavage through reduction and hydrolysis by colonic bacterial enzymes in colon.			
• Polysaccharides	Such systems use substrates damaged by bacteria that are present in the colonic zone. The drug is also coated or incorporated in a substrate matrix			
Time-dependent systems	Drug release is delayed after reaching the small intestine for 3–5 h	This system reduces the dosage and frequency, better patient compliance, avoids the side effect and the drug level is constant at targeted site	The GIT transit time varies between individual which also depends upon the intake of food	[230]
Pressure dependent systems	This method is based on the colon powerful peristaltic waves, which contribute to a momentarily increased luminous pressure	It is mainly used for the drugs which are incorporated in suppository base which is further coated using ethyl cellulose	Only suitable for single unit	[231]
CODES™ technology	This utilizes the merits of polysaccharides, which are destroyed by colon bacteria, combined with pH-sensitive polymer coating	It avoids the problems related with pH dependent and time dependent system.	At desired site the consistency in dissolution of the polymer is lower.	[232]
Microbially triggered the osmotic pump	This approach makes use of the gel forming property of chitosan in acidic environment to generate osmotic pressure and its biodegradation to form pores for colon-specific release of drugs in-situ.	It maintains the drug release in colon for short period as well as constant release of drug for 24 h.	The release rate of drug is affected due to presence of food in GIT.	[233]
Colon targeted micro sponges	The approach uses the pharmaceutical loaded micro sponge's compressibility property to form tablets. Mixtures of HPMC and pectin are used as coating material to produce colon specific delivery of tablets	Micro sponges retain the drug on surface of colon to produce local and targeted action.	It is only used for production of single unit system. The unpredictable chance of disintegration affects the systemic bioavailability of dosage form.	[234]

species were found to be much higher ($P < 0.001$) in rats administered with probiotic containing diet, whereas total coliforms were decreased ($P < 0.001$). It was proposed that the ingested starch acted as a metabolic substrate, thus creating the favourable conditions for *B. lactis* to show its proapoptotic action [140].

3.6. Role of synbiotics in IBDs

IBD is a group of idiopathic, chronic, relapsing-remitting, inflammatory intestinal conditions in which the body is unable to digest food, absorb nutrition and eliminate waste. The most prominent conditions or diseases included in IBDs include UC and Crohn's disease (CD). They cause inflammation in bowel, resulting from the dysregulation of immune system due to dysbiosis. IBD eventually leads to complications like colorectal cancer, anaemia, etc. and can affect some body parts as well (eyes, skin, liver, joints, etc.). UC involves rectal inflammation while CD involves transmural inflammation. So, UC patients are more predisposed to develop colorectal cancer [141].

Symptoms of UC include rectal bleeding, bloody-mucous diarrhoea, and abdominal pain, characterized by relapse and remission periods. Although the pathophysiology of UC is still somewhat uncertain, the relationship between the intestinal microflora and environmental components including stress, diet, hygiene, and antibiotic therapy play an important role in its etiopathology. A continuous colonic involvement results in the reduction of diversity and alteration in the composition of bacteria. Considering gut microbiota as a significant factor involved in the development of IBDs, use of synbiotics is being explored [142].

In IBD, the probiotics inhibit the colonized proliferation of pathogenic microorganisms in colon, and thus, strengthen the immune system and mucosal barrier system of host. Also, probiotics exert anti-inflammatory effects and reduce the pro inflammatory cytokine secretion. The prebiotics, on the other hand, increase the number of probiotics and enhance their activity resulting in the extension in their lifespan. However, the number of studies on the effects of synbiotics, which contain both probiotics and prebiotics together, in UC is quite limited [143].

A randomized, placebo controlled, study was carried out in 40 patients to check the effects of synbiotic therapy in the treatment of patients with active UC having clinical and endoscopic activities. C-reactive protein and the sedimentation values in the synbiotics treated group were observed to decrease significantly while the clinical and endoscopic activities improved [144]. Fujimori et Al. evaluated the effect of synbiotics in treatment of 120 UC patients. The trial compared the effects of probiotics, prebiotics and synbiotics after 2 weeks and 4 weeks, with 40 patients each in probiotic (*B. longum* 2×10^9 CFU), prebiotic (psyllium-8) or symbiotic therapy. Evaluation was done through IBDQs (IBD-Questionnaires). The synbiotic group showed improved IBDQs scores as compared to the prebiotic and probiotic alone groups. Only the symbiotic therapy decreased the C-reactive protein resulting in improved quality of life of UC patients [25]. In another randomized, DB controlled trial on 18 UC patients for one month, the synbiotic treatment (*B. longum* + prebiotic Synergy 1) reduced inflammation and helped in regeneration of epithelial tissue [145].

Amiriani et al. (2020) performed an 8 week long randomized, placebo controlled, DB clinical trial on 60 mild to moderate UC patients to investigate the effect of lactocare (synbiotic) against placebo. Temporal changes in the mean of Simple Clinical Colitis Activity Index (SCCAI) were measured in each group using Chi-Square test. It was observed that the SCCAI decreased significantly on treatment with synbiotics by up to 90–95% as compare to those in the placebo group [146]. The synbiotics treatment comprising of dietary supplements with *Bacillus coagulans* and whole plant sugar cane fibre in mice was found to be more effective in ameliorating the disease than by using the prebiotic and probiotics alone. Synbiotics supplementation significantly reduced the C-protein

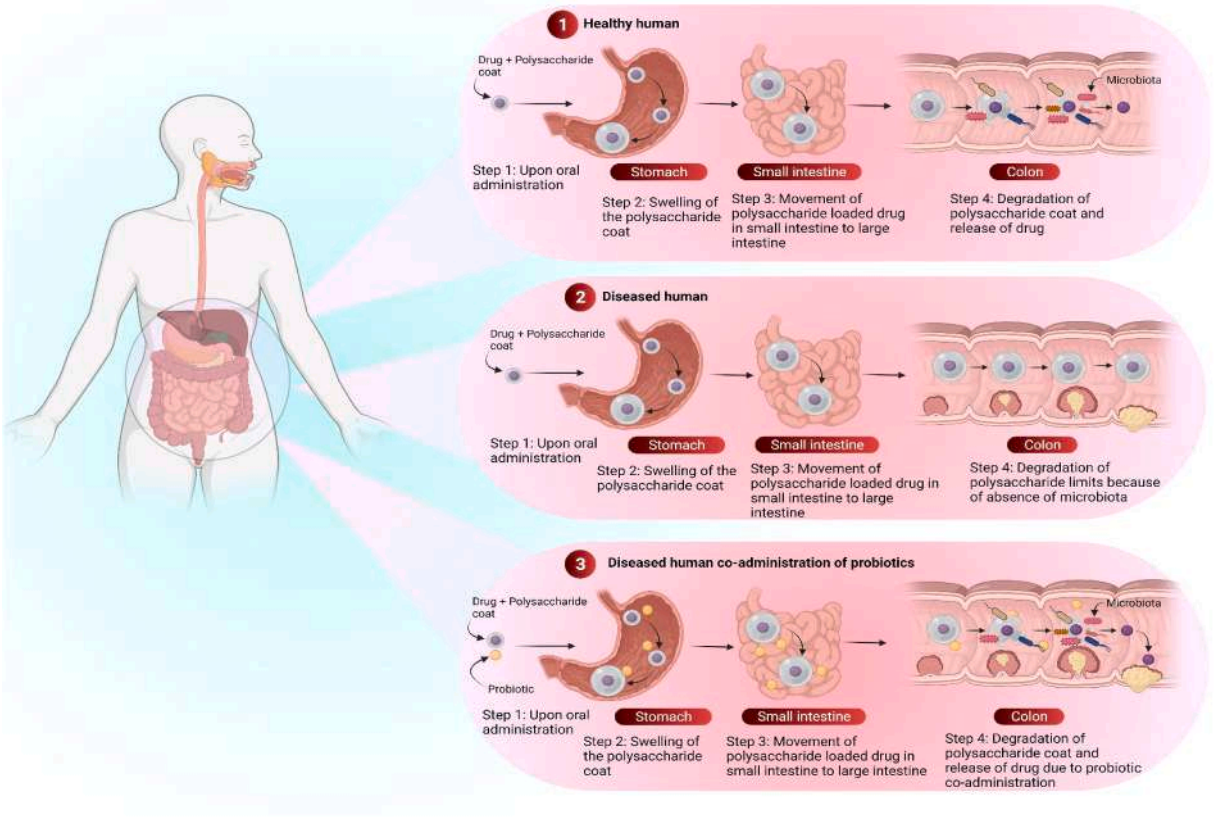

Fig. 7. General mechanism of polysaccharide-based delivery system (7.1); Failure of polysaccharide based controlled drug delivery system (PSBCTDS) in pathological state of colon (7.2); The mechanism of co-administration of probiotic in PSBCTDS (7.3).

levels, prevented the expression of tight junction proteins, and increased the SCFAs. Hence, the synbiotic treatment had a synergistic effect on immune system, epithelial integrity and colonic inflammation [147]. In another randomized, DB, placebo controlled, clinical trial with 35 patients having CD, the immune response against gut microorganisms was checked through administration of synbiotics (*B. longum* and Synergy 1). A significant improvement was observed after collecting clinical scores, TNF-α expression and rectal biopsies at intervals of 0, 3 and 6 months. The disease activity indices and the histological scores also showed a significant reduction with synbiotics treatment [148].

In a similar study, Shinde et al. (2019) used a murine mouse model to determine the effectiveness of the synbiotic combination (*Bacillus coagulans* and whole sugar cane fibre) against inflammation in IBDs. Synbiotics treatment was found to be more effective in reduction disease activity index and histological scores than the control group. The combination treatment also helped in the prevention of alteration in tight junction proteins and C-reactive levels with raised SCFAs [149].

4. Role of synbiotics in drug delivery

As reported above, wide-ranging research has been conducted to explore the therapeutic potential of synbiotics to treat many diseases. In past 5 years, synbiotics have been explored in formulation development of gastric delivery and colon targeted delivery systems as well as carrier material in solidification of formulations such as liquid self-nano emulsifying drug delivery systems (L-SNEDDS) [150] and liquisolid compacts (LSCs) [151] owing to their dual benefits i.e., as nutraceutical/functional food as well as controlled release/enhancing storage stability of formulations.

In one study, the probiotic and prebiotic (ginger extract) were encapsulated in alginate floating beads. The formulated beads remained intact for more than 10 h in the stomach and released synbiotics gradually and over a longer duration from these beads. The combination was reported to be more effective in improving mucus secretion, decreasing ulcer index, decreasing oxidative stress and improving histopathological parameters, compared to probiotic and prebiotic alone (133).

4.1. Role of synbiotics in colon targeted delivery and treatment of IBDs

In oral colon targeted delivery system (CTDS), minimal or no release of drug is desirable from stomach as well as small intestine so that the entire drug reaches the lumen of the large intestine. However, targeting of drugs to colonic site and making their release at pre-determined time intervals is cumbersome because of the diverse physiology of GIT. The sojourn of a formulation through the complete gut with milieus that keep changing in terms of pH, aerobic/anaerobic nature, motility and viscosity without the release of drug is a challenge in itself [152]. Considering these factors, various approaches in the design of oral CTDS have been explored. These include pH dependent system, time-controlled release system, pressure-controlled, osmosis-controlled and microbially triggered delivery systems. The microbially triggered system is further classified into prodrug approach and polysaccharide-based colon targeted delivery system [153]. Various approaches to target the drugs to colon, their advantages and limitations are listed in Table 4.

Among all the reported methods, the polysaccharides-based delivery system has been found suitable for site specific release of drug to colon as polysaccharides are only digested in large intestine by the colonic bacteria. Thus, the basic principle involved in developing this delivery system is based on inclusion of polysaccharides in the matrix and coating the entire formulation by one or more polysaccharides, thus restricting the release of formulation in stomach and intestine. Once the drug

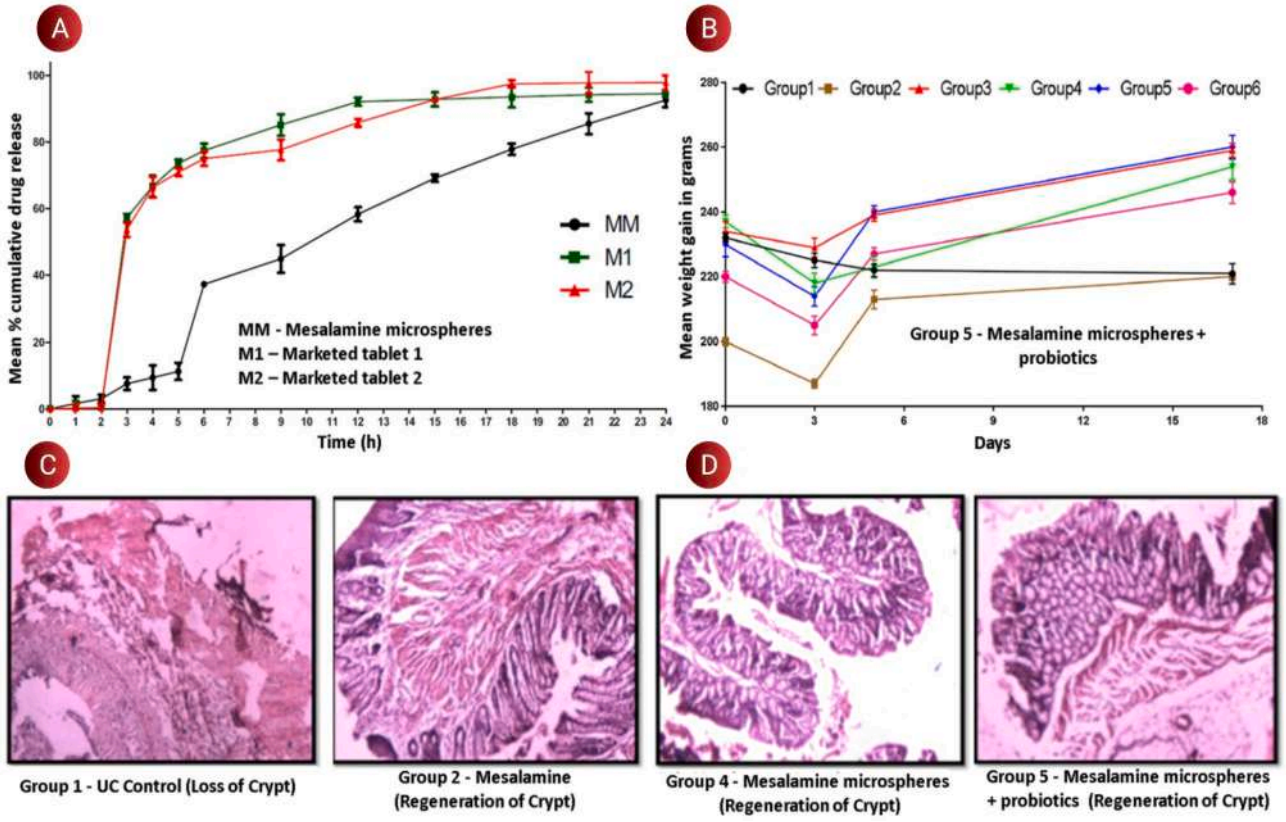

Fig. 8. Effect of synbiotics in mesalamine loaded polysaccharide assisted colon targeted microspheres for UC treatment. Reprinted with permission from Ref. [159] Copyright 2017, Elsevier.

reaches the colon, the gut microbes consume the polysaccharides as they serve as nutrient medium (prebiotic) for microbes and drug release takes place. The mechanism of polysaccharide-based delivery system is shown in Fig. 7.1.

The aforementioned mechanism works well in case of healthy volunteer/animals having non-compromised colon physiology. During the colorectal diseases, the gut dysbiosis takes place and the strength of microbiota gets significantly decreased. During this state the orally administered polysaccharides-based formulation is unable to release the drug even at colonic site. Hence the drug gets excreted out either in intact form or with partial drug release. The schematic diagram in Fig. 7.2. shows the failure of polysaccharide based controlled drug delivery system (PSBCTDS) in pathological state of colon. This failure of polysaccharide-based colon targeted formulations can be overcome by the co-administration/co-formulation of drug-probiotic and polysaccharide based oral CTDS. It can be very well anticipated that when the formulation is co-administered with probiotics, the probiotics would also reach the colon along with the polysaccharide-based formulation and multiply. On subsequent dosing, these replenished microbes will successfully trigger the drug release at colonic site by consuming the polysaccharides. The mechanism of co-administration of probiotic and CTDS is shown in Fig. 7.3. Many polysaccharides have already been tested as colon-specific drug carriers such as chitosan, chondroitin sulfate, dextrans, inulin, pectin, cyclodextrins, and amylose [154]. Various types of bacterial strains present in colonic microflora that are degrade polysaccharide in the gut microflora include *L. acidophilus* LA1, *L. acidophilus* NCFB 1748, *L. GG* (ATCC 53013), *L. casei* Shirota, *S. thermophiles, B. bifidum, L. gasseri* (ADH) and *L. reuteri* [155–158]. In another study, the probiotic and prebiotic (ginger extract) were encapsulated in alginate floating beads.

Though this approach is yet at preclinical stage but it is being explored for different dosage forms and promising results have been achieved in the preclinical studies. Various studies entailing the role of synbiotics in site specific release of drug from oral polysaccharide-based colon targeted drug delivery system are discussed below.

Microspheres of mesalamine were prepared using xanthan gum and guar gum as prebiotics. These were co-administered with probiotic strains *L. rhamnosus, L. acidophilus, Saccharomyces boulardi* and *B. longum*. The prepared microspheres showed superior dissolution profiles as compared to the marketed delayed release formulation of mesalamine in simulated colonic fluid. Less than 5% release of mesalamine was observed from microspheres and its marketed formulations (M1 and M2) in initial 2 h. Upon changing the pH of medium to intestinal pH, the drug release increased to more than 50% from the marketed formulations at the end of third hour. On the other hand, only 7.56% drug was released till 3 h from the prepared microspheres (Fig. 8A and B.) which further increased to 11% in 5 h. This indicated that the microspheres formulated using xanthan and guar gum allowed only 11% drug release till the formulation reached the colon (up to 5 h) and more than 85% drug was delivered to the colon. Addition of probiotics along with microspheres showed excellent therapeutic efficacy in reversing the pathological condition glacial acetic acid induced rat model of UC rats in terms of improved faecal consistency, body weight gain and regeneration of lost crypts in rats treated with mesalamine microspheres and probiotics (Fig. 8C and D.) [159].

In another study, a multiparticulate dosage form consisting of PLGA microcapsules containing prebiotic Bimuno™ incorporated into an alginate–chitosan matrix containing probiotic B. breve was reported. In this formulation, the prebiotic GOS was reported to be released slowly with the initial release occurring over 6 h, and extending up to 288 h. Encapsulation of B. breve in multiparticulates increased the colon targeting as well as increased the viability of probiotics in colon [160].

Chitosan-Ca-alginate microparticles containing synbiotics (*L. casei* 01 + oligofructose enriched inulin) were assessed for their effects in

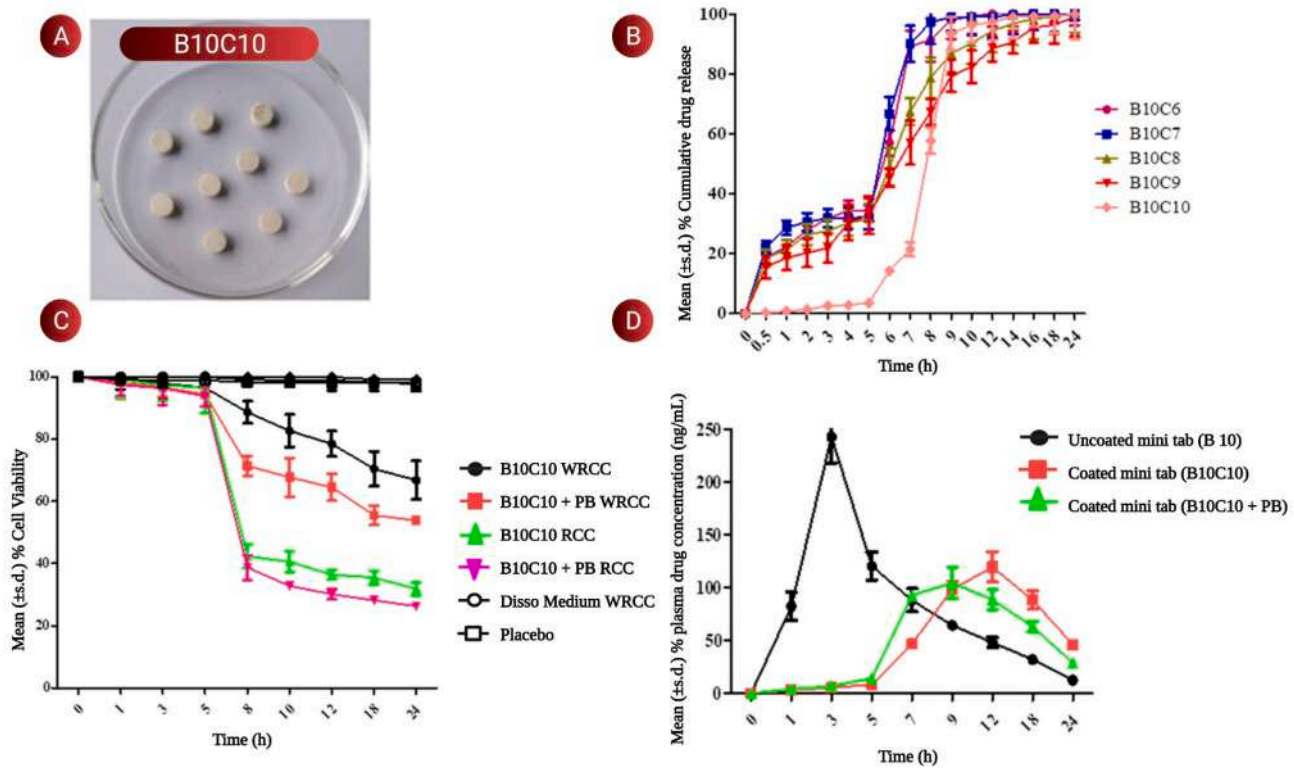

Fig. 9. Effect of probiotics co-administered with guar gum, pectin and Eudragit S100 based colon targeted 5-Fluorouracil loaded mini tablets. Reprinted with permission from Ref. [163] Copyright 2020, Elsevier.

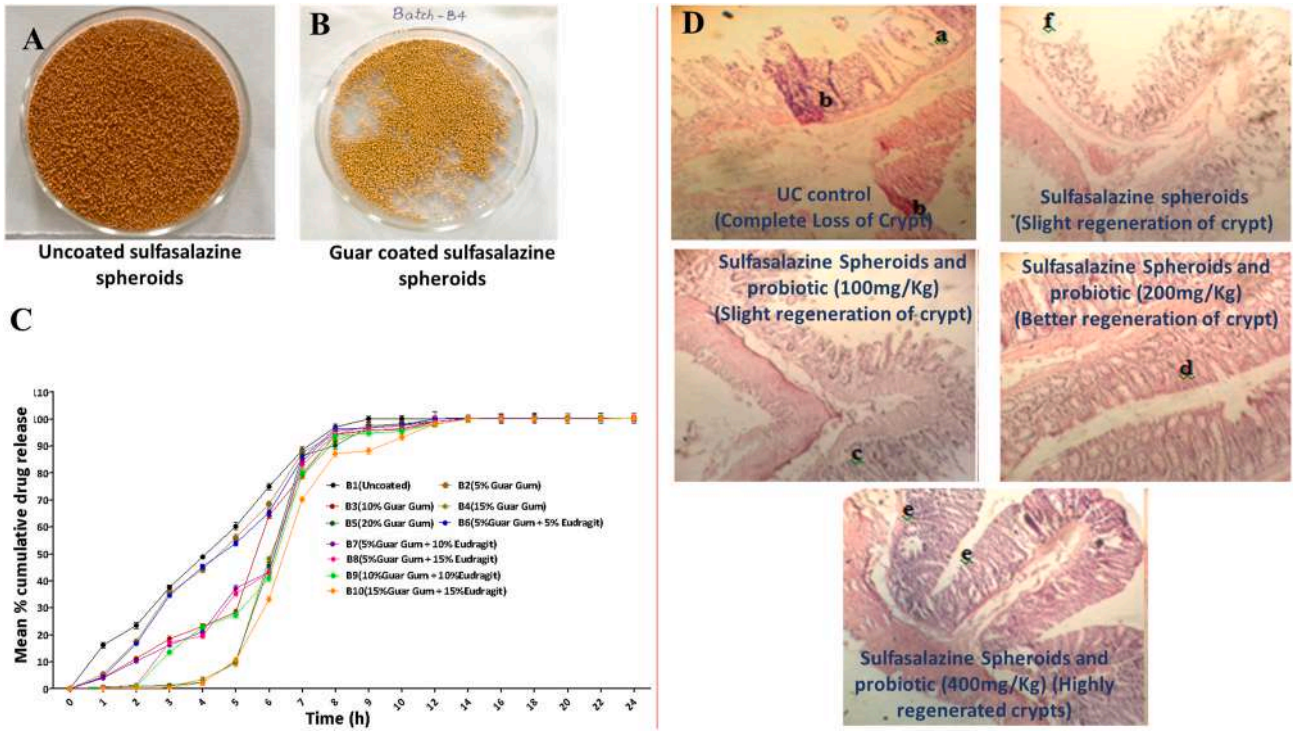

Fig. 10. Role of probiotics with polysaccharide co-administration based CTDS to optimize site specific drug release. Reprinted with permission from Ref. [164] Copyright 2015, Elsevier.

colitis induced rats. The synbiotics treated group showed a reduction in colonic injury and enhanced *Lactobacilli* counts in faeces. The group receiving synbiotics showed the best effect at decreasing lesions and inflammation along with a substantial reduction in myeloperoxidase (MPO) [161]. *Lycium* polysaccharide nanoparticles were prepared by ball milling method, mixed with Bifidobacterium and carbomer and

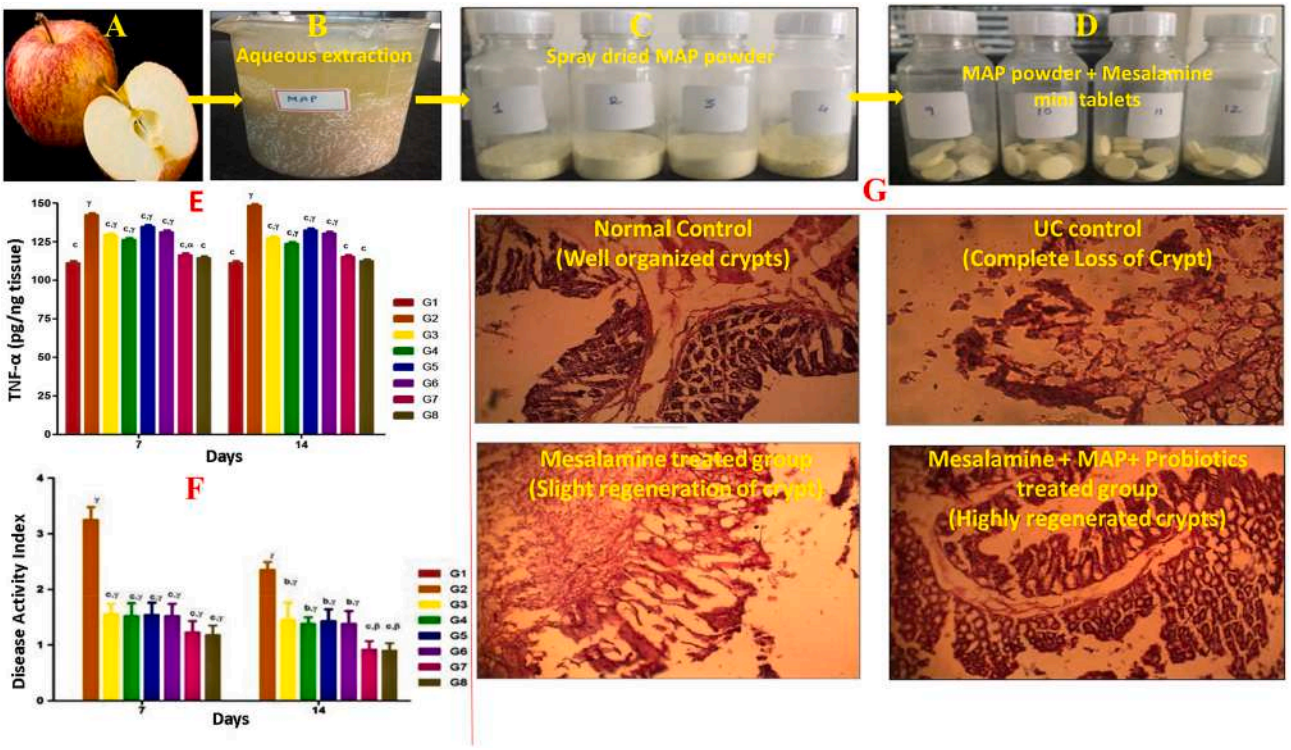

Fig. 11. Efficacy of co-administration of modified apple polysaccharide and probiotics in guar gum-Eudragit S100 based mesalamine mini tablets. Reprinted with permission from Ref. [165] Copyright 2020, Elsevier.

filled into capsules to get the colon targeting synbiotics. The prepared colon targeting formulation was found safe and reliable, met the quality requirements capsules [162].

In another study, the authors developed mini tablets of 5-flurouracil which were coated using pectin, guar gum and Eudragit S100 (Fig. 9A.). These were administered together with probiotics in rats. Less than 10% drug release was obtained from coated tablets till 5 h. Further, between 5 and 10 h, a prompt burst release (100%) was obtained (Fig. 9B.). From cell line studies, more than 90% cells were found to be viable till 5th hour (Fig. 9C.). From the pharmacokinetic studies, reduced and delayed

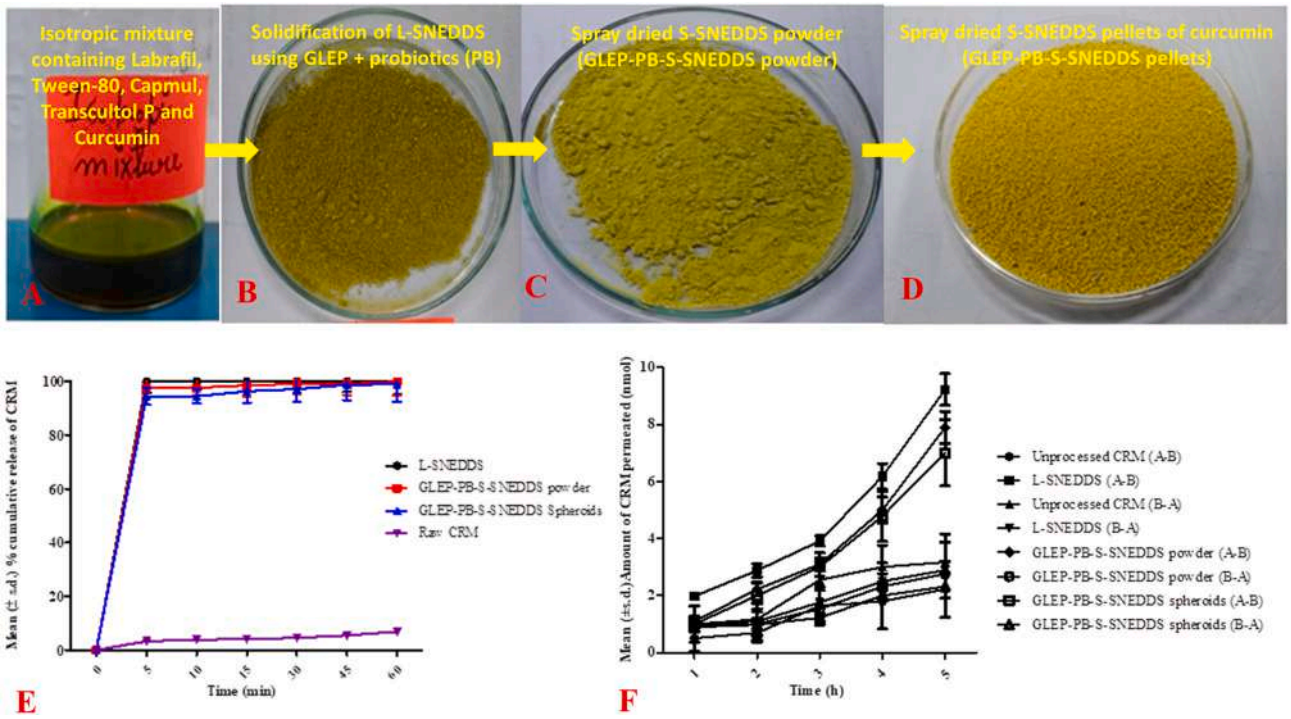

Fig. 12. Case study of curcumin loaded SNEDDS solidified using polysaccharides present in *Ganoderma lucidium* extract powder and probiotics. Reprinted with permission from Ref. [150] Copyright 2021, Elsevier.

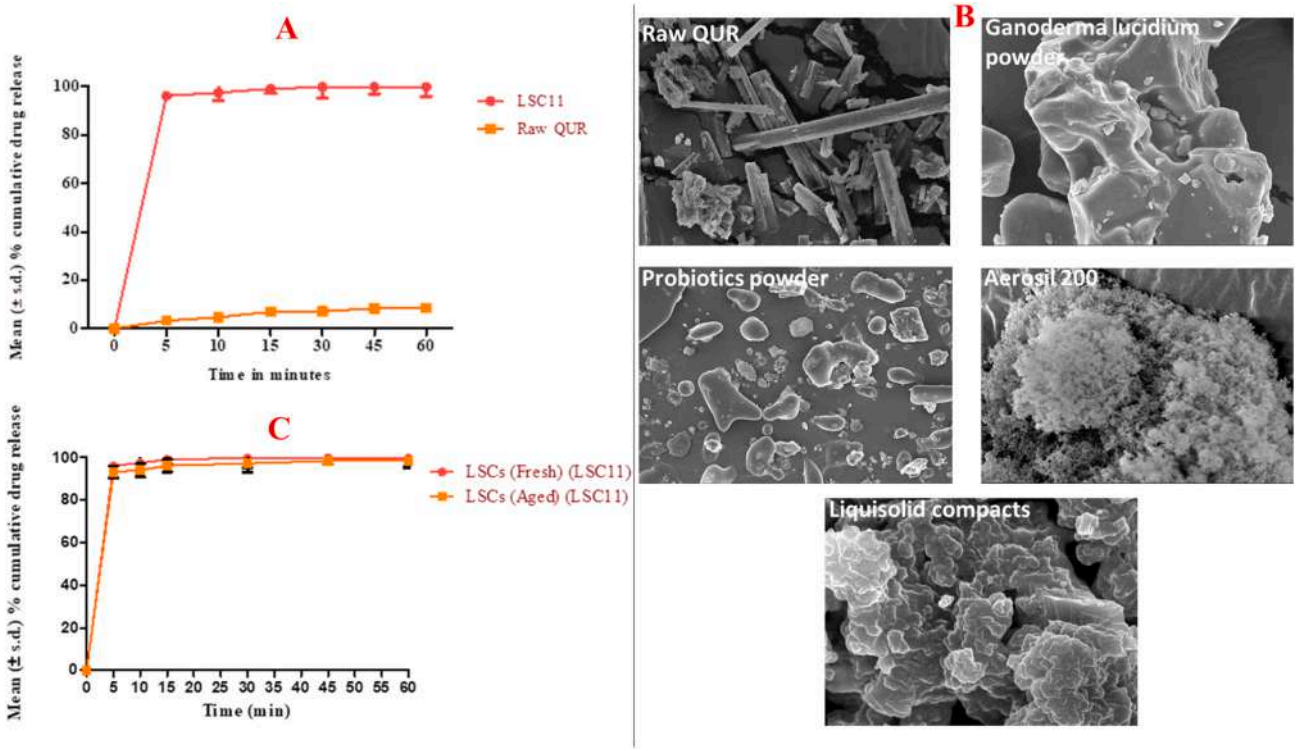

Fig. 13. Case study of quercetin loaded liquisolid formulation solidified using polysaccharides present in *Ganoderma lucidium* extract powder and probiotics. Reprinted with permission from Ref. [151] Copyright 2021, Elsevier.

concentration of drug in plasma was found endorsing the inventive hypothesis of no/low exposure of the non-target sites to the drug (Fig. 9D.) [163].

With similar approach, sulfasalazine spheroids were developed (Fig. 10A and B.). The effectiveness of this CTDS along with supplementation of probiotics was investigated in rats. The results of dissolution studies carried out in simulated colonic fluid indicated that the developed spheroids successfully controlled the drug release as compared to the uncoated spheroids (Fig. 10C.). The drug release was restricted to less than 15% in first 5 h in case of coated spheroids, whereas, the uncoated spheroids failed to restrict the release of sulfasalazine in stomach and intestine. Remarkable reversal of inflammation and crypts damage was noted in group treated with this CTDS along with probiotics. On the other hand, the group treated with spheroid suspension without probiotics, showed moderate inflammation of colonic mucosa and considerable damage of crypts even after two weeks (Fig. 10D.) [164].

Mohanta et al. carried out the aqueous extraction of modified apple polysaccharide (MAP) from apple and solidified using spray drying. Mini tablets containing mesalamine (MES) and MAP were developed (Fig. 11A-D.). The developed mini tablets containing MES and MAP were co-administered with probiotics for targeting the drug to colon of rats induced with UC. MAP was used in the core of the mini tablets while Eudragit S100 and guar gum were used as coating material. Mini tablets of MES and MAP together with probiotics exhibited the most prominent effect in treating UC. The outcomes of disease activity index (Fig. 11E.), antioxidant status, macroscopic scoring, TNF-α (Fig. 11F.) and histopathological examination (Fig. 11G.) indicated that the synbiotic approach provided the maximum beneficial effects [165].

4.2. Use of synbiotics as solid carrier

In a recent study the role of synbiotics has been explored as a solid carrier [150]. Probiotic (Biomix 1) and prebiotic *Ganoderma lucidium* mushroom extract powder (GLEP) was reported for the solidification of L-SNEDDS loaded with curcumin. The SNEDDS formulation was spray dried and ultimately converted into pellets (Fig. 12A–D.). It was seen that the developed powder which was solidified using synbiotics possessed good micromeritic. Further it was seen that solidified formulation could release more than 90% drug in first 5 min. The solidification process of liquid formulation using synbiotics did not significantly affect the droplet size, drug loading, and dissolution indicating the stability of solidified formulation (Fig. 12E.). Moreover, the developed formulation showed significantly higher permeability as compared to that of unprocessed curcumin (Fig. 12F.). Synbiotics, in such applications, play a dual role i.e., as a solid carrier as well as to provide therapeutic benefits.

In another study the authors have explored the use of synbiotics as excipients for preparation of liquisolid compacts (LSCs) of quercetin. Synbiotics acted as adsorbent with a small quantity of A-200 to achieve the desired flow properties [151]. Less than 10% of raw quercetin was released in 60 min whereas more than 90% of quercetin was released from LSCs within 5 min. Moreover, the differential scanning calorimetry (DSC) and powder X-ray diffractometry (PXRD) results showed absence of crystalline peaks of quercetin and scanning electron micrography (SEM) micrographs showed porous appearance indicating that quercetin was effectively dissolved in the LSCs. The LSCs were found to possess good stability during accelerated stability conditions (Fig. 13.). More research focussing on different novel combinations of probiotics and prebiotics is required to further explore the possibilities in this field.

5. Role of microencapsulation and prebiotics in enhancing the stability of probiotics

Certain limitations must be overcome before synbiotics can be utilized as therapeutics. One limitation is the probiotic survival during manufacturing and storage conditions. The viability of probiotic cells in synbiotics must range from 6 to 8 Log CFU/mL. Another major challenge is the transport of live cells to the lower GIT without any considerable reduction of cell viability. The probiotic viability gets reduced inside the

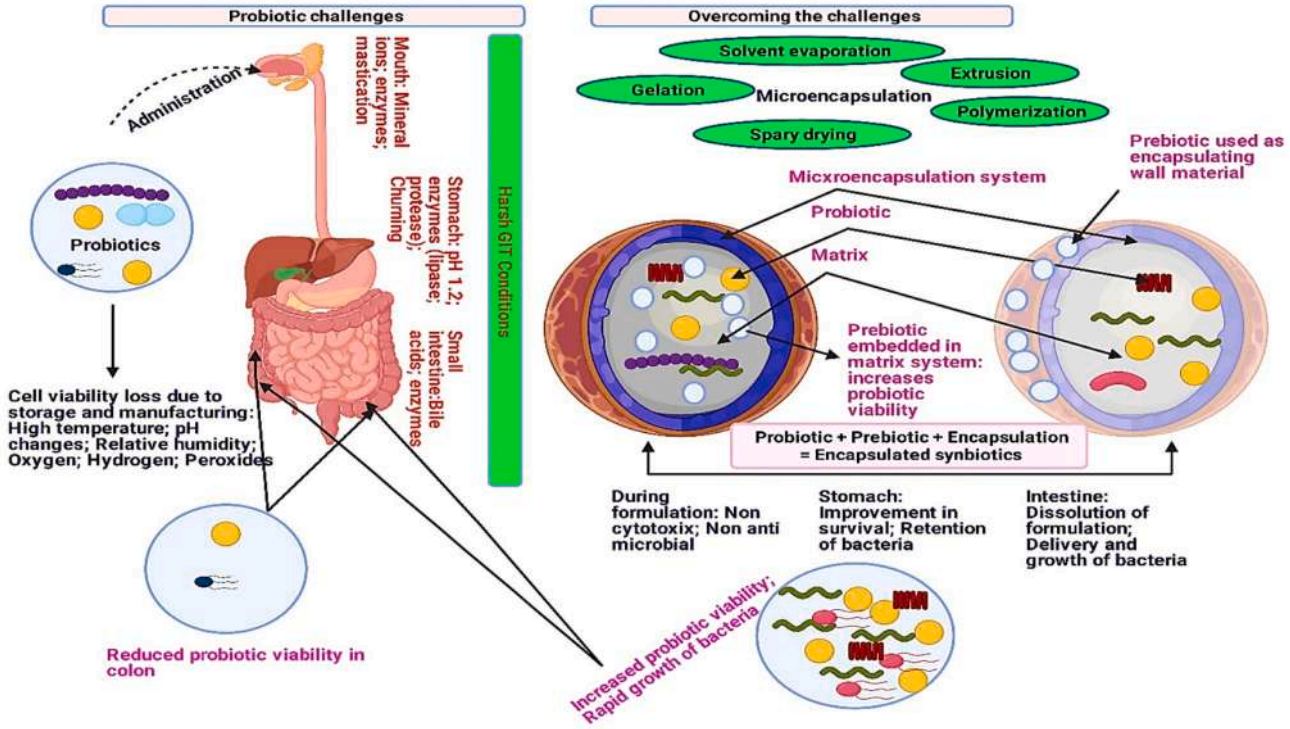

Fig. 14. Figure depicting challenges associated with stability of probiotics and approaches to enhance their viability using microencapsulation technique.

GIT due to the unfavourable environmental factors such as the low pH and bile salts in the digestive system; presence of enzymes in saliva; mastication and presence of minerals in mouth. One of the techniques for enhancing the resilience and live count of probiotic bacteria is by their encapsulation. In this process the core material i.e., probiotic is coated using specific encapsulation material which proves helpful in increasing the probiotic viability by providing a protection barrier to probiotics from injury caused by unfavourable environmental conditions, such as bile salts, heat, and gastric acid [166]. The encapsulation can be carried out using various methods such as solvent evaporation, spray drying and coating, freeze drying, extrusion technique and emulsion technique [167]. Various microencapsulation techniques have been used for enhancing the probiotic viability in synbiotics. Many studies have focussed on impact of prebiotics on the probiotic viability wherein prebiotics have been used as coating agents for probiotics. In other cases, prebiotics have been embedded in the matrix core system to enhance probiotic viability (Fig. 14.).

Nano-emulsions prepared with gum acacia as emulsifier and inulin as prebiotic were used to encapsulate probiotics (*Enterococcus faceium*) Though the stability of probiotic species was enhanced, the average droplet size of the emulsion raised considerably during 60 days stability study [168]. In another study the survival rate of probiotics (72–87%) containing lactic acid bacteria was enhanced in colon by microencapsulation [169]. Wu and Zhang, 2018 conducted a comparative study of microspheres prepared using alginate alone, and combination of alginate as well as prebiotic arabinoxylan. These microspheres were used to encapsulate probiotic *L. plantarum.* The microspheres prepared using arabinoxylan remarkably enhanced the GIT stability (% viability: 51 to74), encapsulation efficiency (2.5 folds), and resistance to bile salt (% viability: 70 to 81) of probiotics. Thus, the synbiotic microspheres can be proposed as perfect carrier for attaining colon targeting of probiotics [170]. In another study, the viability of *Bifidobacterium* species in gastric solution was increased up to 88.29%. In this study, the PLGA nanoparticles of inulin were formulated to lead to its encapsulation. Gum Arabic and alginate were then used as coating material for the nanoparticles [171].

The microencapsulation of probiotic *L. casei* was carried out using spray drying along with complexation of FOS, alginate, and chitosan, and cross-linking using calcium chloride. The product was further freeze dried. The obtained microparticles of average size of 11080 nm were found to possess a positive charge. These increased the cell survival of 10.98 ± 0.11 log CFU/g. The stability of synbiotics was also enhanced in intestinal and gastric juices. Moreover, it was observed that the live cells were released above their therapeutic range (8.31 ± 0.14 log CFU/g) at the colonic pH [172]. Microencapsulation of L. *Plantarum* using spray drying and utilizing whey protein isolate and FOS as the wall material resulted in formation of microcapsules with increased storage stability and protection from harsh GIT conditions [173]. Santos and co-workers microencapsulated *L. acidophilus* La-5, by spray drying technique and utilized inulin as coating material to enhance its GIT viability. A comparative assessment of free and microencapsulated forms was carried out using *in vitro* simulated GIT conditions. It was revealed that the least loss of cell number, after 6 h, was noted in mousse microencapsulated cells (1.3 log cycles), and the highest loss was noted in free cells (7.4 log cycles) [174]. In another study, freeze drying emerged to be more effective encapsulation technique than spray drying of probiotics using FOS and gum Arabic for encapsulation. The maximum probiotic count was noted in case of microcapsules formulated using FOS as coating material (8.74 and 8.75 log CFU/g of lactic acid bacteria and enterococci respectively). Microencapsulated synbiotics and probiotics beneficially modified the faecal microflora of cats, and increased the counts of lactic acid bacteria from 3.65 to 5.07 and 4.87 log CFU/g, respectively [175].

Nunes et al. (2018) encapsulated *L. acidophilus* La-5 using spray drying technique and compared the effect of three prebiotics (inulin, hi-maize and trehalose) as coating materials. Higher encapsulation efficiency was seen in case of hi-maize and inulin (94.26 and 93.12% respectively). However, trehalose was found to be more effective in providing protection against thermal stress. The greatest viability in simulated GIT conditions was noted in case of microparticles with hi-maize. Regarding storage stability, the least loss of bacterial count during 120 days' storage was seen in case of microparticles containing

Table 5
Incorporation of synbiotics in food products.

S. N.	Probiotic	Prebiotic	Food product	Encapsulation method	Outcomes	Reference
1.	*4 Lactobacillli strains*	GOS and lacitol	Yoghurt	Microcapsules using extrusion	Increased probiotic survival	[235]
2.	*L. casei* and "*B. lactis*"	Resistant starch	Ice cream	Calcium alginate beads using emulsification	In case of encapsulated form, the final count after 6 months showed a 0.7 and 1.4 log reduction whereas in case of free form the reduction was 2.9 and 3.4 log for *B. lactis* and *L. casei* respectively	[189]
3.	*L. acidophilus and L. casei*	Resistant starch and inulin	Bread	Microcapsules by emulsion technique	Increased the survival and thermal resistance of probiotics	[188]
4.	*L. acidophilus*	FOS	Yogurt ice cream	Alginate microbeads using gelation technique	In case of encapsulated form, the viable cells reduced to 1 log CFU/g after 60 days whereas in case of control the live counts reduced from ~9.55 to ~7.3 log CFU/g	[236]
5.	*L. acidophilus*	Banana corm stone	Beverage	Freeze dried microcapsules	The cell viability of probiotic was 93.47%. The addition of prebiotic microcapsules produced the best synbiotics beverage with bacterial count 10.4 Log CFU/mL	[166]
6.	*L. bulgaricus and S. thermophilus*	Coloured rice	Yogurt	Spray dried microcapsules	The count of live probiotic bacteria in synbiotics yoghurt was increased than in control group	[237]
7.	*L. Plantarum*	Whey protein + FOS	Noodles	Freeze drying	The cell viability of encapsulated *L. plantarum* was 93.63% and 62.42% in fresh noodles before and after cooking respectively	[238]
8.	*Saccharomyces cerevisiae boulardii*	Alginate–inulin–xanthan gum	Berry juice	Spray drying	Cell viability after storage and fermentation was increased compared to free form (7.59 log10 CFU/mL versus 6.98 log10 CFU/mL, respectively)	[239]
9.	*L. casei*	FOS	Carrot juice	Chitosan-Ca-alginate microparticles using spray drying	Probiotic viability was maintained after 3 months of cold storage (8.1 ± 0.6 log CFU/g)	[240]
10.	*L. plantarum*	Maltodextrin; FOS and pectin	Litchi juice	Spray drying	The best results in terms of yield and viability of encapsulated probiotics were noted in case when maltodextrin were used for coating	[241]
11.	L. lactis *Gh1*	*Synsepalum dulcificum* and gum arabic	Yoghurt	Spray drying	Probiotic in encapsulated forms retained an increased survival, at ~10^7 CFU/mL, when combined into yogurt in contrast to non-microencapsulated form ~10^5 CFU/mL	[242]
12.	*L. plantarum*	Inulin and maltodextrin	Guava juice	Powder using spray drying	Highest viability of 80% for 45 days was obtained at 4 °C and 25 °C	[243]
13.	*L. reuteri*	Alginate inulin (0–1%) and lecithin (0–1%)	Chewing gum	Extrusion	Storage study revealed that, unlike control, the live count of the encapsulated probiotic was retained after 21 days. Inulin and lecithin addition increased the probiotic viability in cell walls	[244]
14.	*L. acidophilus*	Extract of apple skin polyphenol	Milk drink	Co-extrusion	The cell loss in case of co-encapsulated probiotic with aqueous and ethanolic extract of prebiotic was 2.61 and 2.78 log CFU/g of fresh bead, respectively. The loss in case of probiotic encapsulated alone was a little higher whereas the loss in case of non-encapsulated form was much higher	[245]
15.	*L. plantarum LS5*	*Helianthus tuberosus* inulin	Doogh	Alginate beads using emulsification	In case of doogh containing encapsulated bacteria less phase separation and more exopolysaccharides was noted than doogh containing free forms of bacteria. were observed in Inulin incorporation increased the probiotic viability in both encapsulated or free cells in doogh during storage	[246]
16.	*B.lactis (Bb-12)*	Inulin	Cheese	Ca-alginate beads using emulsification	Probiotic survival time and viability was enhanced in low pH of stomach and during storage conditions	[247]
17.	*L. acidophilus TISTR 2365*	Fruiting body of bamboo mushroom	Sweet fermented rice (Khoa-Mak)	Ca- alginate beads using extrusion	Microencapsulation and addition of bamboo mushroom increased the probiotic viability and antioxidant activity	[248]
18.	*L. acidophilusIFO13951 and B. longum ATCC15707*	Red sweet potato	Powdered fermented drink	Spray drying	Microencapsulation efficiency (4.47%) obtained from the formulation containing 75% prebiotic and 25% skim milk exhibited highest encapsulation efficiency. Same formulation showed most enhanced sensory properties with 'neutral' aroma (3.20); 'neutral' flavour (3.3); and 'like' overall (3.50).	[249]
19.	*L. acidophilus*	Whey	Whey drink	Alginate-resistant starch and Eudragit S100	The stability and viability of probiotics increased after encapsulation. Whey drink	[250]

(*continued on next page*)

Table 5 (*continued*)

S. N.	Probiotic	Prebiotic	Food product	Encapsulation method	Outcomes	Reference
				nanoparticles using co-extrusion	containing microencapsulated probiotics showed excellent antioxidant activity	
20.	*L. casei ATCC 39392*	delignified wheat bran	Cream fill cake	Ca- alginate beads using emulsion technique	The viability of microencapsulated *L. casei* was remarkably higher compared to non-encapsulated forms	[251]
21.	*L. casei NCDC 298*	Inulin	Milk chocolate	Ca- alginate beads using emulsion technique	After 60 days storage in refrigerator viable counts remained above 8.0 log CFU/g. No mould or yeast formation was seen.	[252]
22.	*B. animalis*	Inulin	Cocoa cream	Emulsion method	The developed cream was stable after 42 days at 4-degree temp. Addition of inulin to microencapsulated bacteria enhanced the probiotic viability	[253]

trehalose. After storage at room temperature (25 °C) for 120 days, microparticles loaded with all the three prebiotics maintained their number more than the suggested count (>6 log CFU/g) [176]. Moreover, it was seen that encapsulation of *L. casei* using emulsion technique was able to sustain the probiotic viability over 6-months storage at refrigerated conditions in comparison to spray drying technique [177]. Siang and co-workers used the co-extrusion technology for encapsulating *L. rhamnosus* GG. The iso-malto oligosaccharide 3.0% (w/v) was selected as prebiotic due to its growth enhancing effects on probiotic. The integration of prebiotic and coating with poly-L-lysine enhanced the probiotic viability by 3% up to 52% after 2 h of incubation in simulated gastric milieu [178].

In another study, the probiotics were encapsulated in alginate beads containing inulin. The encapsulation showed no effect on probiotic property and their antimicrobial effect. However, addition of inulin to bacteria provided them protection against acidic pH of GIT. Beads containing inulin (5% w/v) showed the most prominent effect against bile-salts protection of bacteria [179]. The probiotic viability was enhanced during freeze drying and subsequent storage conditions (4 °C for 3 months) by encapsulation using prebiotic alginate-fenugreek gum-locust bean gum as matrix system [180]. In another study, *L. plantarum* and *B. lactis* were co-encapsulated using resistant starch or inulin in Ca-alginate/chitosan microcapsules using electro-hydrodynamic atomization (EHDA) technique. Viability studies revealed 5.90 ± 0.3 and 7.19 ± 0.15 log CFU/g of *L. plantarum* and *B. lactis* respectively endured the harsh GIT environment in microcapsules prepared using starch. The L. *plantarum* count loss was efficaciously limited throughout room temperature storage conditions when encapsulated in microcapsules containing inulin since 6.33 ± 0.21 log CFU/g of bacteria were found viable after 3 months storage [181]. *L. acidophilus* NCFM (L-NCFM) was protected in acidic environment by microencapsulation using co-extrusion technique. Coating was done using mannitol and locust bean gum as prebiotics. The viability of encapsulated probiotics ranged from 8.62 log CFU/mL to 6.80 log CFU/mL on storage for 30 days, which fulfilled the condition of least requirement for probiotic (10^6 CFU/mL) [182]. Activity of *B. breve* JCM1192^T and/or raffinose was assessed on epithelial propagation in the rats small and large intestines. Epithelial proliferation in the small intestine, was found to be enhanced only in the group which was fed with combination of encapsulated probiotic and prebiotic whereas, no proliferation was seen in the groups fed with encapsulated probiotic or prebiotic alone. This indicated that the consumed *B. breve* cells utilized raffinose and were activated upon reaching the small intestine, after which they improved epithelial cell proliferation [183].

Microencapsulated inulin containing synbiotic beads prepared using extrusion/ionotropic gelation remained stable in stomach but were degraded to release probiotics in the colon within 3 h [184]. The viability of *L. acidophilus* was increased using microparticles of gelatin alginate and XOS produced by external gelation. Microparticles containing 3% XOS exhibited the highest cell protection in GIT and during

Fig. 15. Marketed synbiotics products.

refrigerated conditions; keeping 97.86 live cell count during storage for 28 days and aiding 87.50% viability after digestion. On the other hand, particles which did not contain XOS exhibited 84.49 and 68.45% viability after storage and after digestion assay, respectively [185]. The authors utilized prebiotic FOS, raffinose and lactulose as coating material for double encapsulation of probiotic *L. acidophilus* ATCC 43121 using dry surface reforming technique (hybridisation). Double microencapsulation proved to be more effective in protecting the probiotics from the acid or heat stress (55 °C) and increasing the probiotic viability up to 20 days storage at 25 and 37 °C. in comparison to non-coated forms [186].

6. Role of microencapsulation and prebiotics in stabilization of probiotics in food products

Incorporation of probiotic as synbiotics into food products is beneficial not only in terms of their intact delivery but also for their therapeutic efficacy. These food products act as carriers for probiotic delivery. But the survival rate of probiotics due to factors such as acidity of the food matrices, storage and manufacturing conditions imposes a limitation for incorporation of these probiotics into food components. Furthermore, the acidic environment of the GIT may prove to be detrimental for their viability. Thus, methods such as enrichment of the matrices with prebiotics and microencapsulation are provide protection of probiotics and enhance their viability [187]. Microencapsulated probiotics have been incorporated into certain foods such as bread [188], ice cream [189] and beverages wherein the probiotic viability is enhanced by using prebiotics, either as coating agents or incorporating the prebiotic in matrix system for encapsulation. A list of these products is summarized in Table 5. Some of the marketed products of synbiotics are shown in Fig. 15.

7. Conclusion

In recent years, the market growth rate of synbiotics has increased significantly and synbiotic market size is forecast to reach $1.8 billion by 2026, growing at a compound annual growth rate of 8.9% during the forecast period 2021–2026 [190]. This is attributed to the rise in understanding the significance of synbiotics as functional foods for health benefits, mainly among the urban consumers. In addition, these synbiotics have been proven beneficial as dietary supplements in clinical studies in treating various diseases. These have been found useful in preventing osteoporosis and lactose intolerance, improving cardiovascular functioning, reduction of inflammation and risk of cancer as well as in management of obesity, hepatic disorders and diabetics. These synbiotics also play an important role in absorption of vital inorganic ions such as calcium, magnesium and phosphorus. Furthermore, these synbiotics have started gaining importance in pharmaceutical industries owing to their health benefits as well as their use as excipients in solidification of emulsions and development of oral targeted drug delivery systems.

Despite these benefits, synbiotics face some major challenges such as their physiochemical instability, particularly that for probiotics, during storage as well as during their sojourn through the GIT. The stringent conditions required during their production, marketing and storage lead to high price of synbiotic products. Overall, these challenge the market potential of synbiotic products. The microencapsulation of synbiotics has been widely reported to address the challenges associated with their gastrointestinal instability. However, more attention is required towards development of unique and economical strategies to stabilize the synbiotics related food supplements and pharmaceutical formulations as the final fate of any product depends upon its stability, which in turn, determines its global acceptability as the storage conditions vary across the globe.

Mutually beneficial combinations need to be optimized for finding the most pertinent combinations for alleviation as well as treatment of specific diseases. Moreover, the regulatory issues related to their marketing should also be taken care. Nevertheless, synbiotics have proven to be of great potential in treating many diseases as dietary supplements and in future it is expected that this potential will be harnessed with the advancement in technology the issues like physicochemical and gastrointestinal instability will be better addressed.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Chewable Tablets of Acacia catechu Extract, an Alternative to Betel (Paan) for Mouth Ulcers: Formulation and In vitro Evaluation

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Abstract

Objective: The objective of the current research work was to prepare chewable tablets having Acacia catechu extract useful for mouth ulcers using a 3^2 factorial design.

Methods: Acacia catechu heartwood extract was prepared using a reported method with some modifications. The extract was characterized using TLC against the catechin marker. Then, drug-excipient interaction studies were carried out. The mixture of drug and excipients was evaluated for pre-compression parameters. With the application of 3^2 factorial design, chewable tablets were prepared using direct compression technique. Prepared tablets were evaluated for post-compression parameters.

Results: In vitro drug release study of the developed formulations was investigated both in intact and crushed form of tablets. Based on the in vitro performance, the best formulations were selected (F6, F7 & F8 from intact and F1, F5 & F9 from the crushed group) and subjected to various kinetic models and evaluated for Chewing Difficulty Index (CDI).

Conclusion: The overall results revealed that the formulated chewable tablets complied with the standards and exhibited the satisfactory performance in terms of drug release, chewing difficulty index and other related parameters.

Keywords: Acacia catechu; catechin; chewable tablet; chewing difficulty index; direct compression; extract.

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REVIEW ARTICLE

Treatment Strategies Against Psoriasis: Principle, Perspectives and Practices

Arya Kadukkattil Ramanunny[1], Sheetu Wadhwa[1], Sachin Kumar Singh[1,*], Deep Shikha Sharma[1], Rubiya Khursheed[1] and Ankit Awasthi[1]

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Abstract: ***Background:*** Psoriasis is a genetically predisposed autoimmune disease mediated by cytokines released by the activated immune cells. It manifests inflammatory, scaly red or white silvery flaky skin which may be a fluid-filled lesion with soreness and itchiness. The prevalence rate of psoriasis is increasing day by day. Despite having such a high prevalence rate, the treatment of psoriasis is still [illegible] well as in the alternative systems of medicine.

Methods: Various bibliographic databases of previously published peer-reviewed research papers were explored and systematic data culminated in terms of various treatment strategies used for the management of psoriasis. The prime focus is given towards modern as well as alternative systems of medicine such as phototherapy, a combination of phototherapy with pharmacotherapy such as Ayurveda, Yoga and naturopathy, Unani, Siddha, and Homeopathy to treat psoriasis.

Results: A comprehensive review of 161 papers, including both research and review articles, was carried out to make the article readily understandable. The pathogenesis including inflammatory mediators and type of psoriasis is discussed before the treatment strategies to understand the pathophysiology of the disease. The uniqueness, procedure, advantages, and limitations of conventional, advanced, and traditional systems of medicine to treat psoriasis are discussed in detail. Emphasis has also been given towards marine sources such as fish oil, marine sponges, and algae.

Conclusion: Although there are many modern and alternative treatment strategies available to treat psoriasis, none of them have been proven to provide complete relief to patients. Moreover, they are associated with certain side effects. In order to overcome them, novel drug delivery systems have been utilized and found effective; however, their stability and safety become the major impediments towards their successful positioning. Traditional and alternative treatment strategies have found to be safe and effective but their use is localized to certain areas. In a nutshell, to achieve successful treatment of pso- [illegible] or the promotion of traditional systems to treat psoriasis.

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1. INTRODUCTION

Psoriasis is an autoimmune disease with marked hyperproliferation and abnormal epidermal differentiation at typical body sites, mostly the extensor surfaces of the elbows, knees, natal cleft, umbilicus, scalp, and nails. It is a chronic inflammatory skin disorder characterized by red or white itchy scales or plaques which might be painful [1]. The condition is triggered by many genetic and environmental factors. It is not only a skin disease as it has a great impact on the physical and psychological quality of life of the person. Some of the comorbidities associated are cardiovascular diseases, obesity, vascular diseases, diabetic mellitus, hypertension, gastrointestinal diseases including inflammatory bowel disease (Crohn's disease), hepatic disease, infection, and mood disorders such as depression, anxiety, suicidal mentation, and stigmatization [2]. It mostly occurs during the early age of 20 to 30 years and in the late age of 50 to 60 years [3]. The condition at this peak age period (teenage) makes the person lose confidence in approaching people due to stigmatization and thereby mentally debilitated and struggle for a secure life.

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REVIEW ARTICLE

Role of Egg Oil in Cosmetics: An Icing on a Cake

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ABSTRACT:

Egg oil is natural oil extracted from standard hen eggs and is a useful constituent of cosmetics and cosmeceuticals, and acts as an anti-oxidant, moisturizer, penetration enhancer, emollient, skin conditioner, and as anti-bacterial agent etc. An important property of egg oil is that, it does not contain egg proteins and thus, is useful for individuals allergic to eggs or its related products especially for therapeutic dermatological applications. Although, with time there is much advancement in the treatment options but these are costly and beyond the budget of common people. Hence, an effective, safe, non-toxic, soothing, and topically applied natural essential oil is the need of the hour. Egg oil is extracted from eggs by different means such as with the help of heat, solvent and cold process. Extracted egg oil can be utilized further in various cosmetics formulations used in hair, skin, nails etc and can be explored in the preparation of various topical delivery systems such as liposomes and microemulsions. Egg oil outlines a light protective barrier on the surface of skin which prevents the loss of moisture, and devoid the clogging of skin pores. Egg oil presence in hair care formulations provides nourishing and conditioning effect. This review article discusses the physicochemical properties of egg oil, its composition, various extraction methods, dermatological applications, commercial sources and patents signifying the tremendous potential of egg oil in cosmetics.

KEYWORDS: Egg oil, hen eggs, nutritional supplement, cosmetics, cosmeceuticals

1. INTRODUCTION:

Egg oil or egg yolk oil is derived from the standard hen's eggs. It is extracted by employing modern technology and available as brand name Oleova, by VAV Life Sciences Pvt. Ltd., Mumbai, India. It consists largely of triglycerides, phospholipids, cholesterol as well as a rich source of poly-unsaturated fatty acids (PUFA), Omega-3, and Omega-6 that are also known as Essential Fatty Acids (EFA). The egg oil composition is resembled closely to the human skin. Egg oil is egg proteins free and therefore may be friendly to egg allegic people, especially in cosmetic preparations (1).

In history, egg oil was used and reported in Unani medicine for the nourishment of hair care. Even chinese medicine employed egg oil in the dermatological treatments of burn wounds, eczema, dermatitis and ulcers, etc (2).

The lipids and oil of eggs is a source of high nutritional and act as a good source of nutraceuticals but their extraction is a tacky process. Because extraction of maximum possible egg oil by keeping them in natural condition is a big question. The composition of egg oil is shown in Figure 1. The clinical trials of egg oil are undergoing by manufacturers in various target areas, but still it can be used safely. The reason is attributed to natural origin of egg oil and it is edible in nature. Extracted egg oil can be utilized further in the preparation of various topical delivery systems such as liposomes, microemulsions and nanoparticles.Fats and fatty acids present in fixed oils and essential oils have been used topically from time immemorial as emollients, protectants and occlusives.

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REVIEW ARTICLE

Vitamin D deficiency, skin, and sunshine: A review

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Abstract

The sunshine vitamin - "Vitamin D" is a subject of interest that attracted researcher's attention in the past few decades. Vitamin D is a fat-soluble vitamin and also a nutrient which increases the calcium absorption and plays an important part in the maintenance of body's immune system and bone formation. However, the deficiency of Vitamin D, also known as hypovitaminosis D, will affect various body parts such as the brain, heart, muscle, immune system, and bones. Thus, the deficiency leads to severe conditions such as osteopenia, osteoporosis, rickets (in children), hypertension, fractures and falls in adults, cancers, diabetes, autoimmune diseases, infections, and neurological disorders. An effective approach for the deficiency prevention is the thorough understanding of Vitamin D sources, Vitamin D serum levels, and deficiency symptoms, linked with different pathological conditions, requirement and maintenance in the body, sunlight exposure duration, and effective treatment dose. Therefore, the present review will lay an emphasis on the role of Vitamin D, the reasons for its deficiency, available sources, and treatment options reported so far.

Key words: Skin, sunlight, Vitamin D, Vitamin D2, Vitamin D3

INTRODUCTION

Nowadays, Vitamin D deficiency affects 50% of the population worldwide. The main reason for its deficiency is the change of lifestyle, i.e. increase in sunscreen use and environmental factors such as reducing the time for exposure to sunlight, which is required for ultraviolet (UV) B rays to induce the production of Vitamin D in the skin. The people who are having black skin require more exposure to the skin for the production of Vitamin D as compared to the white ones.[1] Insufficient Vitamin D will lead to the condition of hypovitaminosis D and the latter is linked with numerous conditions of the body; these are cancer, heart diseases, Type 2 diabetes, probability of stress fractures, autoimmune disease, influenza, and depression.[2]

Vitamin D - Synthesis and Metabolism

Vitamin D is available in two forms: Vitamin D_3 (cholecalciferol) and Vitamin D_2 (ergocalciferol). Vitamin D_3 is a natural occurring form and produced in skin cells from 7-dehydrocholesterol underneath the UV light, and Vitamin D_2 is produced from the natural sterol, i.e. ergosterol.

Vitamin D_3 is metabolized in a two-step non-catalytic process favored by UV light and level of skin pigmentation, into 25-hydroxyvitamin D (25OHD) and then to 1,25-dihydroxyvitamin D ($1,25(OH)_2D$) which is a hormonal form and increases the intestinal calcium absorption, while Vitamin D_2 is metabolized to 25(OH)D in the liver and then to $1,25(OH)_2D$ in the kidneys from ergosterol,[3,4] as shown in Figure 1. The presence of melanin in the skin cells limits the Vitamin D_3 production by blocking the UV rays which affect in the synthesis process. Hence, wearing full sleeves clothes and use of sunscreen too confine its production. In our body, some tissues such as breast, macrophages, colon,

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Efficacy of co-administration of modified apple polysaccharide and probiotics in guar gum-Eudragit S100 based mesalamine mini tablets: A novel approach in treating ulcerative colitis

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Modified apple polysaccharide

ABSTRACT

Modified Apple Polysaccharide (MAP) has been reported to cure colorectal diseases by up-regulating apoptosis and down regulating metastasis. In the present study, mesalamine (MES) and MAP mini tablets have been prepared and co-administered with probiotics to provide site specific release of drug. Probiotics along with MAP, which acts as a prebiotic would replenish the colonic microflora that have been compromised due to colorectal pathology. MES mini tablets were prepared keeping guar gum in the core and coating them with Eudragit S100 and guar gum. The optimized batch was explored for its curative potential on acetic acid induced ulcerative colitis (UC) in rat model with and without administration of probiotic and MAP. The results revealed that the rats treated with the combination of MAP and MES mini tablets along with probiotics show maximum curative potential. It was also observed that MAP mini tablets show better curative potential as compared to probiotics. The results of disease activity index, macroscopic scoring, antioxidant studies, tumour alpha and histopathological examination suggested that the rats treated with combination of MES-MAP mini tablets and probiotics have maximum therapeutic effect followed by MES mini tablets alone, MAP mini tablets alone and probiotics.

1. Introduction

Ulcerative colitis (UC) is a type of chronic inflammatory disease of the colon but with unknown aetiology. The symptoms vary from abdominal pain, bloody diarrhoea and malnutrition. Colonic adenocarcinoma and dysplasia are the most potential threats to ulcer patients, and hence a continuous endoscopic surveillance is required for patient welfare [1]. Approximately 25–30% of the total affected patients require colectomy at certain points of their lives, if treatment with medications goes in vain. Colectomy is considered as the final and permanent cure for UC. General treatments may include one or more medications, balanced diet and intensive moral support to provide an improved quality of life to the patient [1]. Furthermore, people with UC suffer from bloody diarrhoea due to imbalance in gut microflora [2]. As per the available literature the reduction in bacterial count reaches to 30% and in case of lactic acid bacterial subspecies the count reduces from 32 to 18 [2].

The different drugs use for treating ulcerative colitis are mesalamine (MES), sulfasalazine, azathioprine, 6 mercaptopurine etc. MES is an anti-inflammatory agent which is grouped under salicylates and is considered as a first line drug for treating inflammatory bowel disease. It works by diminishing inflammation by the inhibition of prostaglandin production and blocking cyclooxygenase. However, alopecia, cramping, diarrhoea, interstitial nephritis, myalgia, flatulence and nausea are some of the side effects that are observed with long term use of MES [3–5]. Moreover, due to its multiple daily dosing, low adherence to treatment has been reported [6]. This causes frequent flares of colitis that ultimately increases medical costs hence, the strategies to improve drug's adherence are greatly in demand [7]. Hence, delivery of MES to colon, by passing the upper GI regions is expected to reduce the side effects.

In past few decades oral colon specific drug delivery systems (CSDDS) have attracted researchers as they help in delivering the drug at the targeted site. CSDDS works on the principle of protection of drugs' degradation in stomach and small intestine due to presence of acidic environment and other digestive enzymes and successfully delivering the drugs at colonic site [7]. Keeping this objective various delivery

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Research Article

INFLUENCE OF FORMULATION PARAMETERS ON DISSOLUTION RATE ENHANCEMENT OF ACYCLOVIR USING LIQUISOLID FORMULATION

KUBOTA MWAKA HAZEMBA, JIVAN JYOTI, SHEETU WADHWA*, SANANDA SOM, SOUVIK MOHANTA, ANKIT KUMAR YADAV, BIMLESH KUMAR, VARUN GARG, DEEP SHIKHA SHARMA, RUBIYA KHURSHEED, MONICA GULATI, SACHIN KUMAR SINGH

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ABSTRACT

Objective: The objective of this research work is to explore the use of liquisolid technique in enhancement of acyclovir dissolution rate. This current study was planned to assess the impact of different formulation variables, such as non-volatile liquid type and concentrations of acyclovir on its dissolution rates profile.

Method: Acyclovir liquisolid tablets were prepared with Tween 60 (liquid vehicle), Microcrystalline cellulose PH 102 (acted as a carrier to turn liquid medication into free-flowing powder) and Syloid XDP (coating material). *In vitro*, drug dissolution rate of liquisolid formulations of acyclovir was performed and compared with pure acyclovir drug using USP dissolution apparatus (Type II) for 60 min at a paddle speed of 50 rpm and filled with 900 mL of distilled water.

Results: The dissolution study showed that 94.1% of the drug was released in 60 min of ratio 10 while only 66% of the pure drug acyclovir was released in 60 min. Hence, present work concluded that the acyclovir dissolution rate profile has been improved with the formation of liquisolid formulations..

Conclusion: From the present study, it may be ratified that the drug dissolution rate of acyclovir has been improved with the utilization of liquisolid formulations approach.

Keywords: Acyclovir, Dissolution, Non-volatile liquid, Liquisolid tablets.

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INTRODUCTION

Nowadays, low solubility is the principle issue related with new drug moieties which should be overcome. The vast majority of the procedures that have prevailing with regard to conquering this issue are spray drying, micronization approach, β-cyclodextrins inclusion complexes, solid dispersions, and eutectic mixtures. However, among all, the feasible and financially savvy is liquisolid system. In liquisolid strategy, the drug is held in a solubilized state and molecularly dispersed in a liquid which helps to achieve the improved drug dissolution [1].

To overcome the bioavailability issue of poorly soluble drugs due to insufficient dissolution rate, numerous techniques are being utilized. Hydrophilic polymers as solubility enhancers are used in various approaches which perform through many ways in the development of various techniques such as cosolvency and inclusion complexes which provide numerous advantages toward formulation development. However, most of the time, during storage, these techniques show stability issues, and low industrial viability and are not commercially successful. Moreover, these techniques suffered with limitations such as hygroscopic and sticky mass which lead to poor flow of powders [2].

Liquisolid technology emerged as a new drug delivery system, differentiated due to its features and potential to deliver numerous drugs. These systems have drawn the attention of pharmaceutics scientists and scholars in the area of poorly soluble drugs for their solubility enhancement and controlled dissolution profile as per the formulation requisite [2]. This technology is patented by Spireas *et al.*, in 1999, and is a simple process of physical mixing with selected excipients which turns it into a free-flowing, dry powder. The main formulation components of liquisolid systems are non-volatile liquid vehicle, a carrier, and a coating material. Moreover, as per the objectives and need of the study, sometimes other excipients such as disintegrants or superdisintegrants are used [3-5].

One of the anticipated mechanisms for the enhance the dissolution rate of the drug from the liquisolid compact mass is the wettability of the latter in the dissolution media. The component that helps in the wetting of drug particles in liquisolid system is the non-volatile solvent [6]. These solvent systems reduce the interfacial tension which was exist between the tablet surface and selected dissolution medium which results in increase effective surface area and wettability for dissolution [7]. Due to this fact, these liquisolid compacts show improved dissolution profile and enhanced bioavailability of poorly soluble drugs. The release of drug from these liquisolid compacts is mainly depends on few parameters, such as drug characteristics, type of carrier, and the type of liquid vehicle used in the formulation. Therefore, these should be optimized, and its effect on dissolution rate should be evaluated [8,9].

The main components of liquisolid systems are carrier material, coating material, non-volatile solvent, and disintegrant. Carrier material such as various grades of sorbitol, cellulose, lactose, and starch holds sufficient adsorption property [10]. Coating materials are highly adsorptive and usually in very fine particle size range. These are various grades of colloidal silica such as Syloid

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Abstract No. RTBS-O-09

SAFE AND EFFECTIVE TOPICAL DELIVERY OF BETAMETHASONE NANO-COLLOIDAL CARRIER: DERMATOLOGICAL AND HISTOLOGICAL EVALUATION

SHEETU WADHWA[1], BHUPINDER SINGH[2], SACHIN SINGH[1], DEEP SHIKHA SHARMA[1], AND OM PRAKASH KATARE[2]

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Abstract

Topical steroidal anti-inflammatory drugs like Betamethasone (BTM) are highly prevalent in a number of skin disorders and diseases, because of their proven efficacy. But they simultaneously suffer from serious limitations such as skin atrophy, irritation and improper absorption. Hence forth, this piece of work aims to evolve a formulation strategy based on the principles of novel drug delivery and exploitation of unique biomaterials like phospholipids to circumvent the problems while improving the overall performance. Various nano-lipoidal carriers loaded with BTM were prepared by thin-film hydration (liposomes and transfersomes). The developed carriers were characterized for micromeritics, morphology and drug loading potential. Topical delivery attributes were evaluated by skin permeation and skin retention studies employing mice skin. Developed systems were evaluated for bio-safety studies on shaved Laca mice. Anti-psoriatic activity was also performed on mouse-tail model and evaluated histopathologically. The formulations were assessed for stability as per ICH guidelines. All the colloidal carriers were found to lie in the optimum size with almost regular geometry. The topical drug transport characteristics of colloidal carriers were found to be significantly better than the marketed product. These carriers were better tolerated as no histopathological changes were observed in the mice skin samples. These offered better skin retention in the form of micro-reservoirs for prolonged stay. A significant enhancement in the anti-psoriatic activity was observed *vis-a-vis* the commercial one. The developed carriers were found to stabilize the entrapped drug. These delivery benefits may scientifically be attributed to the changes brought forth in the drug molecules at the level of physicochemical characteristics which would modify the kinetics leady to improved pharmacodynamic efficacy. Nano-colloidal carriers based on biocompatible lipids were able to increase the safety and efficacy of BTM.

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Application Details	
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DATE OF FILING	29/11/2019
APPLICANT NAME	**Lovely Professional University**
TITLE OF INVENTION	A NOVEL FORMULATION OF 5-FLUOROURACIL FOR TREATING DIABETIC RETINOPATHY
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E-MAIL (As Per Record)	dip@lpu.co.in
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REQUEST FOR EXAMINATION DATE	--
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FIELD OF INVENTION	CHEMICAL
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Recent advances in intraocular and novel drug delivery systems for the treatment of diabetic retinopathy

Deep Shikha Sharma , Sheetu Wadhwa , Monica Gulati , Arya kr , Ankit Awasthi , Sachin Kumar Singh , Rubiya Khursheed , Leander Corrie , Nitin Chitranshi , Vivek Kumar Gupta & Sukriti Vishwas

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Current Strategies and Future Perspective for the Effective Treatment of Diabetic Retinopathy

Deep Shikha Sharma[1], Sachin Kumar Singh[1], Divya Thakur[2], Arya K.R[1], Rubiya Khursheed[1] and Sheetu Wadhwa[1,*]

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Abstract: Diabetes retinopathy (DR) is one of the main complications due to diabetes. DR will damage the retinal capillaries and block them, which causes the loss of vision. Different drugs and therapies are used for the treatment and prevention of the DR. The most commonly used treatment is laser technology and combination therapy, along with some drugs. But these drugs possess side effects in the form of cataract, glaucoma, and complete blindness of the eye. The main strategy to overcome In DR, medicines with minimum side effects or maximum therapeutic effects are used. This article emphasizes the current strategy used for the treatment of DR with allopathic as well as herbal drugs.

Keywords: Diabetes, diabetic retinopathy, herbal drugs, laser therapy.

1. INTRODUCTION

1.1. Diabetes Mellitus (DM)

Diabetes mellitus (DM) or Diabetes is a chronic condition in which the human body is not able to produce insulin or it is unable to use insulin which leads to hyperglycemia, *i.e.* the increase in the plasma glucose concentration (as shown in Table 1 below). This hormone is released from the β cells of the islets of langerhens of pancreas which helps in the breakdown of glucose. Hyperglycemia causes other complications like CVS, neuropathy, nephropathy, blindness, diabetes retinopathy, *etc.* [1].

DM is mainly of three types: Type 1, 2, and gestational diabetes. Other types are monogenic and secondary diabetes. The causes and symptoms of the aforementioned types are shown in Table **2**.

1.2. Diabetes Retinopathy (DR)

In India, DR is a major complication of DM which leads to visual impairment. DR damages the retinal capillaries and blocks them, which causes loss of vision. DR is mainly of two types- proliferative and non-proliferative. Further classification of DR is shown in Fig. **1**.

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Proliferative DR

In this type of DR, there is abnormal growth of the blood vessels (also known as neovascularization), which are fragile and can break easily, this causes sudden vision loss.

Non-proliferative DR

This type of DR involves different stages such as-

i. **Mild DR**- Damage of small retinal blood vessels and balloon-like swelling called microaneurysms

ii. **Moderate DR**- Blockage of retinal blood vessels, which causes a decrease in oxygen and nutrient supply to the retina.

iii. **Severe DR**- Retinal ischemia due to the blockage of a large number of retinal blood vessels [1, 2].

2. PATHOPHYSIOLOGY

The pathophysiology of DR is shown in Fig. **2** below.

Diabetes affects millions of people worldwide. It also affects other body parts like kidney, heart, foot, and eyes. Increased level of glucose *i.e.* Hyperglycemia is the main cause of diabetes, which leads to metabolic dysfunction and activation of chronic, low-grade inflammatory signaling which has an important role in DR. Along with medication, some changes in lifestyle helps in the maintenance of BP and sugar level, other activities like daily exercise, taking a balanced

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RESEARCH ARTICLE

RJPT

Estimation of 5-fluorouracil by high-performance liquid Chromatography Reversed-phase Validated method

Deep Shikha Sharma, Sheetu Wadhwa, Sachin Kumar Singh*, Arya Ramanunny, Rajan Kumar
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ABSTRACT:

The main intention of the current work is the development of a suitable, simple, precise, reproducible, and accurate high-performance liquid chromatography reversed-phase validated method which can be used for the estimation of 5-Fluorouracil.The estimation was done by high-performance liquid chromatography (HPLC) method. In this suitable stationary phase and optimum composition of the mobile phase was selected which provides good resolution and short run time for the estimation of 5-Fluorouracil (5-FU). Stationary phase used was Nucleodur C18 column (Reverse phase, 250mm × 4.6mm i.d., particle having 5 micron size) and mobile phase consists of combination of Ortho-Phosphoric Acid (OPA) (0.5%) and methanol having ratio 95:5, v/v were used in an isocratic mode of elution. The mobile phase used with a flow rate of 0.8 mL/min and volume of the injection for 5-FU was 20µL. The eluent observed at 266nm for measurement of 5-FU. The validation of this method was carried out with the help of various parameters such as sensitivity, selectivity, system suitability, precision (inter-day and intra-day), accuracy, and linearity according to International Conference on Harmonization guidelines i.e. ICH Q2 (R1). The 5-FU showing retention time at 7.2 min. The responses showing linearity in the concentration range between 2-10µg/mL with correlation coefficient 0.99. The % mean recovery of 5-FU was calculated at three different levels i.e Lower Quality control Concentration (LQC), Middle Quality control Concentration (MQC), High Quality control Concentration (LQC) whose results falls within the range i.e. 95% to 105%, which indicates the accuracy of this method. The % relative standard deviation (RSD) precision (intraday and intermediate) at 3 different levels was < 2% which indicated the precision of this method. LOD i.e. Limit of Detection and LOQ i.e. Limit of Quantification was found to be 0.870277 and 2.637202 respectively for 5-FU.

KEYWORDS: 5-Fluorouracil, RP-HPLC, ICH Guidelines, System suitability, Accuracy, Precision.

INTRODUCTION:

5-Fluorouracil (5-FU) is a pyrimidine analogue (shown in Figure 1 below) and used as anticancer and antimetabolite. 5-FU blocks the thymidylate synthetase enzyme activity which is required for the conversion of deoxyuridylic acid to thymidylic required for synthesis of DNA [1,4]. For estimation of 5-FU a suitable, simple, precise, reproducible, and accurate high-performance liquid chromatography (HPLC) reversed-phase (RP) validated method is required which can be further used for the bioanalytical estimation of 5-FU.

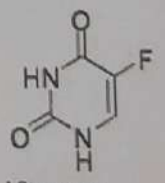

Figure 1: 5-FU Chemical Structure

The importance of analytical method validation in drug development process:

HPLC developed and validated method helps in identification as well as quantification of compounds. As per GMP (Good Manufacturing Practice) requirements different pharmaceutical companies have validation policy to determine the purity of the drug, presence of relative substances, and stability profile of drug [2]. The current validated method also helps in estimation of drug in biological fluids during

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Chewable Tablets of Acacia catechu Extract, an Alternative to Betel (Paan) for Mouth Ulcers: Formulation and In vitro Evaluation

Pooja Bhardwaj [1], Kodati Shailendra [2], Deep Shikha Sharma [2], Amit Sharma [1], Mangesh Pradeep Kulkarni [2], Sachin Kumar Singh [2], Vrinder Pal Singh [2], Gurvinder Singh [2], Pardeep Kumar [2], Rajesh Kumar [2]

Affiliations

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Abstract

Objective: The objective of the current research work was to prepare chewable tablets having Acacia catechu extract useful for mouth ulcers using a 3^2 factorial design.

Methods: Acacia catechu heartwood extract was prepared using a reported method with some modifications. The extract was characterized using TLC against the catechin marker. Then, drug-excipient interaction studies were carried out. The mixture of drug and excipients was evaluated for pre-compression parameters. With the application of 3^2 factorial design, chewable tablets were prepared using direct compression technique. Prepared tablets were evaluated for post-compression parameters.

Results: In vitro drug release study of the developed formulations was investigated both in intact and crushed form of tablets. Based on the in vitro performance, the best formulations were selected (F6, F7 & F8 from intact and F1, F5 & F9 from the crushed group) and subjected to various kinetic models and evaluated for Chewing Difficulty Index (CDI).

Conclusion: The overall results revealed that the formulated chewable tablets complied with the standards and exhibited the satisfactory performance in terms of drug release, chewing difficulty index and other related parameters.

Keywords: Acacia catechu; catechin; chewable tablet; chewing difficulty index; direct compression; extract.

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Current Drug Delivery, 2020, *17*, 52-73

REVIEW ARTICLE

Treatment Strategies Against Psoriasis: Principle, Perspectives and Practices

Arya Kadukkattil Ramanunny[1], Sheetu Wadhwa[1], Sachin Kumar Singh[1,*], Deep Shikha Sharma[1], Rubiya Khursheed[1] and Ankit Awasthi[1]

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Abstract: ***Background:*** Psoriasis is a genetically predisposed autoimmune disease mediated by cytokines released by the activated immune cells. It manifests inflammatory, scaly red or white silvery flaky skin which may be a fluid-filled lesion with soreness and itchiness. The prevalence rate of psoriasis is increasing day by day. Despite having such a high prevalence rate, the treatment of psoriasis is still [illegible] well as in the alternative systems of medicine.

Methods: Various bibliographic databases of previously published peer-reviewed research papers were explored and systematic data culminated in terms of various treatment strategies used for the management of psoriasis. The prime focus is given towards modern as well as alternative systems of medicine such as phototherapy, a combination of phototherapy with pharmacotherapy such as Ayurveda, Yoga and naturopathy, Unani, Siddha, and Homeopathy to treat psoriasis.

Results: A comprehensive review of 161 papers, including both research and review articles, was carried out to make the article readily understandable. The pathogenesis including inflammatory mediators and type of psoriasis is discussed before the treatment strategies to understand the pathophysiology of the disease. The uniqueness, procedure, advantages, and limitations of conventional, advanced, and traditional systems of medicine to treat psoriasis are discussed in detail. Emphasis has also been given towards marine sources such as fish oil, marine sponges, and algae.

Conclusion: Although there are many modern and alternative treatment strategies available to treat psoriasis, none of them have been proven to provide complete relief to patients. Moreover, they are associated with certain side effects. In order to overcome them, novel drug delivery systems have been utilized and found effective; however, their stability and safety become the major impediments towards their successful positioning. Traditional and alternative treatment strategies have found to be safe and effective but their use is localized to certain areas. In a nutshell, to achieve successful treatment of pso- [illegible] or the promotion of traditional systems to treat psoriasis.

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Keywords: Psoriasis, novel drug delivery systems, phototherapy, alternative systems, marine sources, homeopathy.

1. INTRODUCTION

Psoriasis is an autoimmune disease with marked hyperproliferation and abnormal epidermal differentiation at typical body sites, mostly the extensor surfaces of the elbows, knees, natal cleft, umbilicus, scalp, and nails. It is a chronic inflammatory skin disorder characterized by red or white itchy scales or plaques which might be painful [1]. The condition is triggered by many genetic and environmental factors. It is not only a skin disease as it has a great impact on the physical and psychological quality of life of the person. Some of the comorbidities associated are cardiovascular diseases, obesity, vascular diseases, diabetic mellitus, hypertension, gastrointestinal diseases including inflammatory bowel disease (Crohn's disease), hepatic disease, infection, and mood disorders such as depression, anxiety, suicidal mentation, and stigmatization [2]. It mostly occurs during the early age of 20 to 30 years and in the late age of 50 to 60 years [3]. The condition at this peak age period (teenage) makes the person lose confidence in approaching people due to stigmatization and thereby mentally debilitated and struggle for a secure life.

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REVIEW ARTICLE

Role of Egg Oil in Cosmetics: An Icing on a Cake

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ABSTRACT:

Egg oil is natural oil extracted from standard hen eggs and is a useful constituent of cosmetics and cosmeceuticals, and acts as an anti-oxidant, moisturizer, penetration enhancer, emollient, skin conditioner, and as anti-bacterial agent etc. An important property of egg oil is that, it does not contain egg proteins and thus, is useful for individuals allergic to eggs or its related products especially for therapeutic dermatological applications. Although, with time there is much advancement in the treatment options but these are costly and beyond the budget of common people. Hence, an effective, safe, non-toxic, soothing, and topically applied natural essential oil is the need of the hour. Egg oil is extracted from eggs by different means such as with the help of heat, solvent and cold process. Extracted egg oil can be utilized further in various cosmetics formulations used in hair, skin, nails etc and can be explored in the preparation of various topical delivery systems such as liposomes and microemulsions. Egg oil outlines a light protective barrier on the surface of skin which prevents the loss of moisture, and devoid the clogging of skin pores. Egg oil presence in hair care formulations provides nourishing and conditioning effect. This review article discusses the physicochemical properties of egg oil, its composition, various extraction methods, dermatological applications, commercial sources and patents signifying the tremendous potential of egg oil in cosmetics.

KEYWORDS: Egg oil, hen eggs, nutritional supplement, cosmetics, cosmeceuticals

1. INTRODUCTION:

Egg oil or egg yolk oil is derived from the standard hen's eggs. It is extracted by employing modern technology and available as brand name Oleova, by VAV Life Sciences Pvt. Ltd., Mumbai, India. It consists largely of triglycerides, phospholipids, cholesterol as well as a rich source of poly-unsaturated fatty acids (PUFA), Omega-3, and Omega-6 that are also known as Essential Fatty Acids (EFA). The egg oil composition is resembled closely to the human skin. Egg oil is egg proteins free and therefore may be friendly to egg allegic people, especially in cosmetic preparations (1).

In history, egg oil was used and reported in Unani medicine for the nourishment of hair care. Even chinese medicine employed egg oil in the dermatological treatments of burn wounds, eczema, dermatitis and ulcers, etc (2).

The lipids and oil of eggs is a source of high nutritional and act as a good source of nutraceuticals but their extraction is a tacky process. Because extraction of maximum possible egg oil by keeping them in natural condition is a big question. The composition of egg oil is shown in Figure 1. The clinical trials of egg oil are undergoing by manufacturers in various target areas, but still it can be used safely. The reason is attributed to natural origin of egg oil and it is edible in nature. Extracted egg oil can be utilized further in the preparation of various topical delivery systems such as liposomes, microemulsions and nanoparticles.Fats and fatty acids present in fixed oils and essential oils have been used topically from time immemorial as emollients, protectants and occlusives.

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REVIEW ARTICLE

Vitamin D deficiency, skin, and sunshine: A review

Sheetu Wadhwa[1], Deep Shikha Sharma[1], Meenu Mehta[1], Divya Thakur[2], Sanchit Mahajan[3], Sachin Kumar Singh[1], Saurabh Satija[1]

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Abstract

The sunshine vitamin - "Vitamin D" is a subject of interest that attracted researcher's attention in the past few decades. Vitamin D is a fat-soluble vitamin and also a nutrient which increases the calcium absorption and plays an important part in the maintenance of body's immune system and bone formation. However, the deficiency of Vitamin D, also known as hypovitaminosis D, will affect various body parts such as the brain, heart, muscle, immune system, and bones. Thus, the deficiency leads to severe conditions such as osteopenia, osteoporosis, rickets (in children), hypertension, fractures and falls in adults, cancers, diabetes, autoimmune diseases, infections, and neurological disorders. An effective approach for the deficiency prevention is the thorough understanding of Vitamin D sources, Vitamin D serum levels, and deficiency symptoms, linked with different pathological conditions, requirement and maintenance in the body, sunlight exposure duration, and effective treatment dose. Therefore, the present review will lay an emphasis on the role of Vitamin D, the reasons for its deficiency, available sources, and treatment options reported so far.

Key words: Skin, sunlight, Vitamin D, Vitamin D2, Vitamin D3

INTRODUCTION

Nowadays, Vitamin D deficiency affects 50% of the population worldwide. The main reason for its deficiency is the change of lifestyle, i.e. increase in sunscreen use and environmental factors such as reducing the time for exposure to sunlight, which is required for ultraviolet (UV) B rays to induce the production of Vitamin D in the skin. The people who are having black skin require more exposure to the skin for the production of Vitamin D as compared to the white ones.[1] Insufficient Vitamin D will lead to the condition of hypovitaminosis D and the latter is linked with numerous conditions of the body; these are cancer, heart diseases, Type 2 diabetes, probability of stress fractures, autoimmune disease, influenza, and depression.[2]

Vitamin D - Synthesis and Metabolism

Vitamin D is available in two forms: Vitamin D_3 (cholecalciferol) and Vitamin D_2 (ergocalciferol). Vitamin D_3 is a natural occurring form and produced in skin cells from 7-dehydrocholesterol underneath the UV light, and Vitamin D_2 is produced from the natural sterol, i.e. ergosterol.

Vitamin D_3 is metabolized in a two-step non-catalytic process favored by UV light and level of skin pigmentation, into 25-hydroxyvitamin D (25OHD) and then to 1,25-dihydroxyvitamin D ($1,25(OH)_2D$) which is a hormonal form and increases the intestinal calcium absorption, while Vitamin D_2 is metabolized to 25(OH)D in the liver and then to $1,25(OH)_2D$ in the kidneys from ergosterol,[3,4] as shown in Figure 1. The presence of melanin in the skin cells limits the Vitamin D_3 production by blocking the UV rays which affect in the synthesis process. Hence, wearing full sleeves clothes and use of sunscreen too confine its production. In our body, some tissues such as breast, macrophages, colon,

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Efficacy of co-administration of modified apple polysaccharide and probiotics in guar gum-Eudragit S100 based mesalamine mini tablets: A novel approach in treating ulcerative colitis

Souvik Mohanta [a], Sachin Kumar Singh [a,*], Bimlesh Kumar [a], Monica Gulati [a], Rajesh Kumar [a], Ankit Kumar Yadav [a], Sheetu Wadhwa [a], Jivan Jyoti [a], Sananda Som [a], Kamal Dua [b,c,d,e], Narendra Kumar Pandey [a]

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ARTICLE INFO

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Keywords:
Ulcerative colitis
Mesalamine
Modified apple polysaccharide

ABSTRACT

Modified Apple Polysaccharide (MAP) has been reported to cure colorectal diseases by up-regulating apoptosis and down regulating metastasis. In the present study, mesalamine (MES) and MAP mini tablets have been prepared and co-administered with probiotics to provide site specific release of drug. Probiotics along with MAP, which acts as a prebiotic would replenish the colonic microflora that have been compromised due to colorectal pathology. MES mini tablets were prepared keeping guar gum in the core and coating them with Eudragit S100 and guar gum. The optimized batch was explored for its curative potential on acetic acid induced ulcerative colitis (UC) in rat model with and without administration of probiotic and MAP. The results revealed that the rats treated with the combination of MAP and MES mini tablets along with probiotics show maximum curative potential. It was also observed that MAP mini tablets show better curative potential as compared to probiotics. The results of disease activity index, macroscopic scoring, antioxidant studies, tumour alpha and histopathological examination suggested that the rats treated with combination of MES-MAP mini tablets and probiotics have maximum therapeutic effect followed by MES mini tablets alone, MAP mini tablets alone and probiotics.

1. Introduction

Ulcerative colitis (UC) is a type of chronic inflammatory disease of the colon but with unknown aetiology. The symptoms vary from abdominal pain, bloody diarrhoea and malnutrition. Colonic adenocarcinoma and dysplasia are the most potential threats to ulcer patients, and hence a continuous endoscopic surveillance is required for patient welfare [1]. Approximately 25–30% of the total affected patients require colectomy at certain points of their lives, if treatment with medications goes in vain. Colectomy is considered as the final and permanent cure for UC. General treatments may include one or more medications, balanced diet and intensive moral support to provide an improved quality of life to the patient [1]. Furthermore, people with UC suffer from bloody diarrhoea due to imbalance in gut microflora [2]. As per the available literature the reduction in bacterial count reaches to 30% and in case of lactic acid bacterial subspecies the count reduces from 32 to 18 [2].

The different drugs use for treating ulcerative colitis are mesalamine (MES), sulfasalazine, azathioprine, 6 mercaptopurine etc. MES is an anti-inflammatory agent which is grouped under salicylates and is considered as a first line drug for treating inflammatory bowel disease. It works by diminishing inflammation by the inhibition of prostaglandin production and blocking cyclooxygenase. However, alopecia, cramping, diarrhoea, interstitial nephritis, myalgia, flatulence and nausea are some of the side effects that are observed with long term use of MES [3–5]. Moreover, due to its multiple daily dosing, low adherence to treatment has been reported [6]. This causes frequent flares of colitis that ultimately increases medical costs hence, the strategies to improve drug's adherence are greatly in demand [7]. Hence, delivery of MES to colon, by passing the upper GI regions is expected to reduce the side effects.

In past few decades oral colon specific drug delivery systems (CSDDS) have attracted researchers as they help in delivering the drug at the targeted site. CSDDS works on the principle of protection of drugs' degradation in stomach and small intestine due to presence of acidic environment and other digestive enzymes and successfully delivering the drugs at colonic site [7]. Keeping this objective various delivery

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INNOVARE ACADEMIC SCIENCES
Knowledge to Innovation

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Research Article

INFLUENCE OF FORMULATION PARAMETERS ON DISSOLUTION RATE ENHANCEMENT OF ACYCLOVIR USING LIQUISOLID FORMULATION

KUBOTA MWAKA HAZEMBA, JIVAN JYOTI, SHEETU WADHWA*, SANANDA SOM, SOUVIK MOHANTA, ANKIT KUMAR YADAV, BIMLESH KUMAR, VARUN GARG, DEEP SHIKHA SHARMA, RUBIYA KHURSHEED, MONICA GULATI, SACHIN KUMAR SINGH

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Received: 28 February 2018, Revised and Accepted: 16 March 2018

ABSTRACT

Objective: The objective of this research work is to explore the use of liquisolid technique in enhancement of acyclovir dissolution rate. This current study was planned to assess the impact of different formulation variables, such as non-volatile liquid type and concentrations of acyclovir on its dissolution rates profile.

Method: Acyclovir liquisolid tablets were prepared with Tween 60 (liquid vehicle), Microcrystalline cellulose PH 102 (acted as a carrier to turn liquid medication into free-flowing powder) and Syloid XDP (coating material). *In vitro*, drug dissolution rate of liquisolid formulations of acyclovir was performed and compared with pure acyclovir drug using USP dissolution apparatus (Type II) for 60 min at a paddle speed of 50 rpm and filled with 900 mL of distilled water.

Results: The dissolution study showed that 94.1% of the drug was released in 60 min of ratio 10 while only 66% of the pure drug acyclovir was released in 60 min. Hence, present work concluded that the acyclovir dissolution rate profile has been improved with the formation of liquisolid formulations..

Conclusion: From the present study, it may be ratified that the drug dissolution rate of acyclovir has been improved with the utilization of liquisolid formulations approach.

Keywords: Acyclovir, Dissolution, Non-volatile liquid, Liquisolid tablets.

 DOI: http://dx.doi.org/10.22159/ajpcr.2018.v11s2.28537

INTRODUCTION

Nowadays, low solubility is the principle issue related with new drug moieties which should be overcome. The vast majority of the procedures that have prevailing with regard to conquering this issue are spray drying, micronization approach, β-cyclodextrins inclusion complexes, solid dispersions, and eutectic mixtures. However, among all, the feasible and financially savvy is liquisolid system. In liquisolid strategy, the drug is held in a solubilized state and molecularly dispersed in a liquid which helps to achieve the improved drug dissolution [1].

To overcome the bioavailability issue of poorly soluble drugs due to insufficient dissolution rate, numerous techniques are being utilized. Hydrophilic polymers as solubility enhancers are used in various approaches which perform through many ways in the development of various techniques such as cosolvency and inclusion complexes which provide numerous advantages toward formulation development. However, most of the time, during storage, these techniques show stability issues, and low industrial viability and are not commercially successful. Moreover, these techniques suffered with limitations such as hygroscopic and sticky mass which lead to poor flow of powders [2].

Liquisolid technology emerged as a new drug delivery system, differentiated due to its features and potential to deliver numerous drugs. These systems have drawn the attention of pharmaceutics scientists and scholars in the area of poorly soluble drugs for their solubility enhancement and controlled dissolution profile as per the formulation requisite [2]. This technology is patented by Spireas *et al.*, in 1999, and is a simple process of physical mixing with selected excipients which turns it into a free-flowing, dry powder. The main formulation components of liquisolid systems are non-volatile liquid vehicle, a carrier, and a coating material. Moreover, as per the objectives and need of the study, sometimes other excipients such as disintegrants or superdisintegrants are used [3-5].

One of the anticipated mechanisms for the enhance the dissolution rate of the drug from the liquisolid compact mass is the wettability of the latter in the dissolution media. The component that helps in the wetting of drug particles in liquisolid system is the non-volatile solvent [6]. These solvent systems reduce the interfacial tension which was exist between the tablet surface and selected dissolution medium which results in increase effective surface area and wettability for dissolution [7]. Due to this fact, these liquisolid compacts show improved dissolution profile and enhanced bioavailability of poorly soluble drugs. The release of drug from these liquisolid compacts is mainly depends on few parameters, such as drug characteristics, type of carrier, and the type of liquid vehicle used in the formulation. Therefore, these should be optimized, and its effect on dissolution rate should be evaluated [8,9].

The main components of liquisolid systems are carrier material, coating material, non-volatile solvent, and disintegrant. Carrier material such as various grades of sorbitol, cellulose, lactose, and starch holds sufficient adsorption property [10]. Coating materials are highly adsorptive and usually in very fine particle size range. These are various grades of colloidal silica such as Syloid

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Vol 11, Issue 3, 2018

INNOVARE ACADEMIC SCIENCES

Online - 2455-3891
Print - 0974-2441

Abstract No. RTBS-O-09

SAFE AND EFFECTIVE TOPICAL DELIVERY OF BETAMETHASONE NANO-COLLOIDAL CARRIER: DERMATOLOGICAL AND HISTOLOGICAL EVALUATION

SHEETU WADHWA[1], BHUPINDER SINGH[2], SACHIN SINGH[1], DEEP SHIKHA SHARMA[1], AND OM PRAKASH KATARE[2]

[1]School of Pharmaceutical Sciences, Lovely Professional University, Phagwara – 144411, Punjab, India.[2]Drug Delivery Research Group, University Institute of Pharmaceutical Sciences, Panjab University, Chandigarh 160 014, India.Email: sheetu.21001@lpu.co.in

Abstract

Topical steroidal anti-inflammatory drugs like Betamethasone (BTM) are highly prevalent in a number of skin disorders and diseases, because of their proven efficacy. But they simultaneously suffer from serious limitations such as skin atrophy, irritation and improper absorption. Hence forth, this piece of work aims to evolve a formulation strategy based on the principles of novel drug delivery and exploitation of unique biomaterials like phospholipids to circumvent the problems while improving the overall performance. Various nano-lipoidal carriers loaded with BTM were prepared by thin-film hydration (liposomes and transfersomes). The developed carriers were characterized for micromeritics, morphology and drug loading potential. Topical delivery attributes were evaluated by skin permeation and skin retention studies employing mice skin. Developed systems were evaluated for bio-safety studies on shaved Laca mice. Anti-psoriatic activity was also performed on mouse-tail model and evaluated histopathologically. The formulations were assessed for stability as per ICH guidelines. All the colloidal carriers were found to lie in the optimum size with almost regular geometry. The topical drug transport characteristics of colloidal carriers were found to be significantly better than the marketed product. These carriers were better tolerated as no histopathological changes were observed in the mice skin samples. These offered better skin retention in the form of micro-reservoirs for prolonged stay. A significant enhancement in the anti-psoriatic activity was observed *vis-a-vis* the commercial one. The developed carriers were found to stabilize the entrapped drug. These delivery benefits may scientifically be attributed to the changes brought forth in the drug molecules at the level of physicochemical characteristics which would modify the kinetics leady to improved pharmacodynamic efficacy. Nano-colloidal carriers based on biocompatible lipids were able to increase the safety and efficacy of BTM.

Certificate

CHITKARA COLLEGE OF PHARMACY
CHITKARA UNIVERSITY, PUNJAB

6th International Conference on

"Pharmacy Practice Clinical Research and Pharmacovigilance Trends-2019" (PPCRPV-2019)"

THIS IS TO CERTIFY THAT

Dr./ Mr./Ms. Deep Shikha Sharma

OF

Lovely Professional University

has participated in the International Conference **"PPCRPV-2019"** on 8th and 9th November, 2019. He/She presented an Oral Presentation/E-Poster Presentation on

Estimation of 5-Fluorouracil by High Performance liquid chromatography and its validation.

Dr. MADHU CHITKARA
CHIEF PATRON
Vice Chancellor | Chitkara University

Dr. SANDEEP ARORA
CHAIRMAN & CONVENER
PPCRPV-2019 |Chitkara University

Dr. THAKUR GURJEET SINGH
ORGANISING SECRETARY
PPCRPV-2019 | Chitkara University

Dr. MANJINDER SINGH
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Dr. Kishore Gnana Sam
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Indian Pharmaceutical Association

CHITKARA UNIVERSITY

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PHARMACY PRACTICE SUMMIT-2019
Collaborative Summit
Advanced Clinical and Community Pharmacy Services in Qualitative Therapeutics and Healthcare Delivery: The serious missing link"

This is to certify that ~~Dr./Mr.~~/Ms.
Deep Shikha Sharma
of
Lovely Professional University.
has participated in the **"Pharmacy Practice Summit-2019"**
on 19th to 20th April, 2019
as ~~Student~~ / Research Scholar/Faculty Participant.
~~He~~/ She presented an E-Poster Presentation on

CURRENT STRATEGIES AND FUTURE PERSPECTIVE FOR THE EFFECTIVE TREATMENT OF DIABETIC RETINOPATHY

Dr. MADHU CHITKARA PATRON Vice Chancellor Chitkara University, Punjab	**Dr. SANDEEP ARORA** Convener & Chairman PPS-2019 Chitkara University, Punjab	**Program Collaborators** PCI, IPA, APTI, IHPA PPS-2019 Chitkara University, Punjab	**Dr. THAKUR GURJEET SINGH** Organising Secretary PPS-2019 Chitkara University, Punjab

14TH CHANDIGARH SCIENCE CONGRESS

CHASCON-2020

Certificate of Appreciation

This certificate is awarded to Mr./Mrs./Dr./Prof. ***Deepshika Sharma*** for Oral Presentation in 14th Chandigarh Science Congress jointly organised by Panjab University and Chandigarh Region Innovation and Knowledge Cluster on 17th-19th December, 2020.

Presentation entitled- ***Formulation of 5-Flurouracil loaded nanostructured lipid carriers for the management of diabetes retnopat***

Section- ***Pharmaceutical sciences***

Prof. Desh Deepak Singh	Prof. Ravinder Kumar Singla	Prof. Sandeep Sehajpal
Coordinator	DUI	Co-coordinator

Certificates No. 173666

Certificate of Participation

This is to certify that Prof./Dr./Mr./Ms. DEEPSHIKHA SHARMA

has Presented E-poster/Oral presentation on Estimation of 5-Fluorouracil by High performance Liquid Chromatography & its validation.

presentation in International Conference of Pharmacy (ICP-2019) on the theme of " Pharmacy: Realigning the foc on health" held on 13 -14th September 2019 organized by School of Pharmaceutical Sciences, Lovely Professio University, Punjab in a collabration with Indian Pharmacy Graduates' Assocaition (IPGA) Phagwara chapter.

Date of Issue: 14-09-2019
Place of Issue: Phagwara (India)

Prepared by
(Administrative Officer-Records)

Dr. Bimlesh Kumar
Organizing Secretary

Dr. Monica Gulati
Chairperson, Local Organizing Com

DIVISION OF RESEARCH AND DEVELOPMENT

[Under the Aegis of Lovely Professional University, Jalandhar-Delhi G.T. Road, Phagwara (Punjab)]

Certificate No.240338

Certificate of Participation

This is to certify that **Ms. Deep Shikha Sharma** of **Lovely Professional University, Phagwara, Punjab, India** has presented paper on **Formulation Development, Characterization and In vitro Evaluation of 5- Fluorouracil Loaded Nanostructured Lipid Carriers** in the **International Conference on Materials for Emerging Technologies (ICMET-21)** held on February 18-19, 2022, organized by Department of Research Impact and Outcome, Division of Research and Development, Lovely Professional University, Punjab.

Date of Issue: 16-03-2022
Place: Phagwara (Punjab), India

Prepared by
(Administrative Officer-Records)

Dr. Vipul Srivastava
Convener
(ICMET-21)

Dr. Manish Vyas
Organizing Secretary
(ICMET-21)

Dr. Chander Prakash
Co-Chairperson
(ICMET-21)

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Certificate of Participation

This is to certify that

DEEP SHIKHA SHARMA

of *LOVELY PROFESSIONAL UNIVERSITY*

has actively participated in AICTE sponsored

Short Term Training Programme

Organized by COP-BELA from 11th -16th January, 2021.

Dr. Shailesh Sharma
Convener

16th January, 2021
Certificate No.: COP-BELA/2021/STTP/I/71

Dr. Monika Gupta
Org. Secretary

UG-Pharmacy | 60th in 2020 | AICTE-CII Platinum Category | U/S 2(f) | Permanent Affiliated | 9001:2015

Anton Paar

Deep Shikha Sharma

Lovely Professional University, Punjab

attended the webinar on

Basics of Rheology for Pharmaceutical Applications

Date: June 11, 2021

Time: 11:30 to 12:30 hours

Contents

The training provided the essential knowledge for:

- What is Rheology
- Rheometer and Measuring Geometries
- Rotation Tests
- Oscillation Tests
- Powder Rheology

Date of issue: June 15, 2021

Mr. Mayank Varshney
Application Specialist
Characterisation Division
Anton Paar India Pvt. Ltd.

FACULTY OF APPLIED MEDICAL SCIENCES

[Under the Aegis of Lovely Professional University, Jalandhar-Delhi G.T. Road, Phagwara (Punjab)]

Certificate No. 235741

Certificate of Participation

This is to certify that Mr./Ms. **Deep Shikha Sharma** S/O,D/O,W/O **Mr. Tarsem Lal Sharma**

student of **School of Pharmaceutical Sciences** Registration No. **41700243**

pursuing **PhD** participated in the **National Pharmacovigilance Week-2021**

on the theme of **"Pharamacovigilance: A step towards patient safety"** held from **17-09-2021** to **23-09-2021**

organized by **Adverse Drug Reaction Monitoring Centre (AMC) and Medical Device Adverse Event Monitoring Centre (MDMC)**

in association with School of Pharmaceutical Sciences at Lovely Professional University, Punjab.

Date of Issue : 12-11-2021
Place of Issue: Phagwara (India)

Prepared by
(Administrative Officer-Records)

Dr. Bimlesh Kumar
Organizing Secretary

Monica Gulati

Prof. (Dr.) Monica Gulati
Executive Dean
LFAMS

SWAMI VIVEKANAND GROUP OF INSTITUTES

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WOMEN FORUM

Workshop

on

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18th January, 2021

Organized by

Swami Vivekanand College of Pharmacy, Banur,& Indian Pharmacy Graduate Association - Women Forum

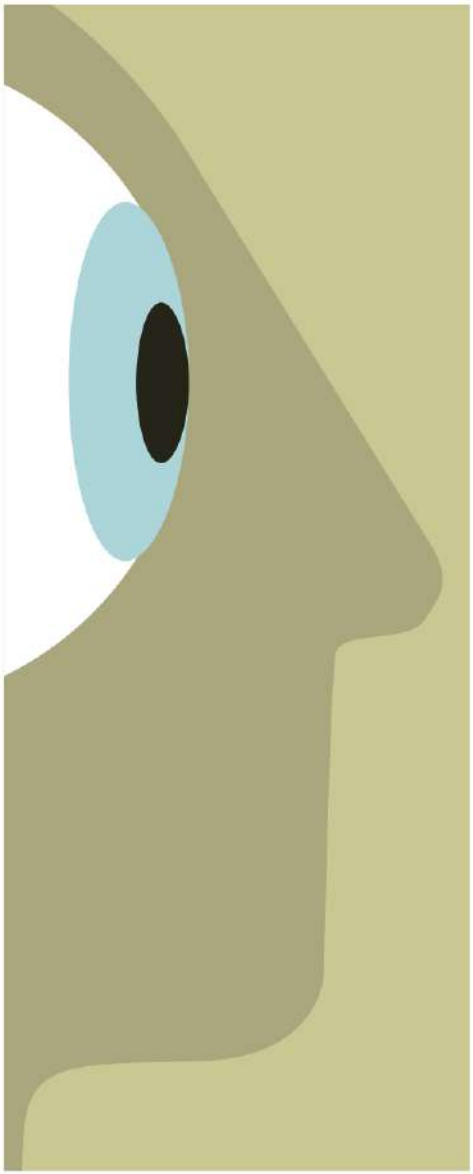
Certificate of Participation

This is to certify that Dr./Mr/Ms Deep Shikha Sharma of Lovely Professional University participate as delegate during the workshop held on 18th January, 2021.

Ms. Prabhjot Kaur
Coordinator

Ms. Sonia Pahuja
Organising Secretary

Dr. Prerna Sarup
Punjab State Coordinator IPGA-WF



Researcher Academy On Campus

Certificate of Attendance

ELSEVIER

This certifies that

Deep Shikha Sharma

has attended the following

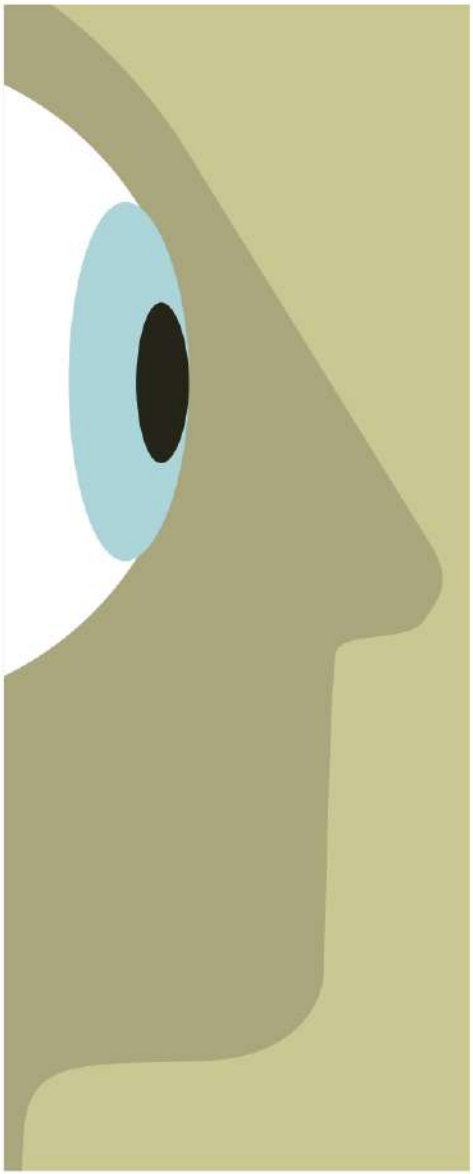
Two Day Webinar: Navigating the Pathways of Research Publishing in Scopus-indexed journals- Day 1

at Research and Consultancy Cell, Vidya Prabodhini College (VPCCECM), Goa,
on Friday 24 September, 2021

Presented by Vishal Gupta Sr. Consultant, Elsevier, Dr. Sumit Narula Deputy Dean Research, Amity University Gwalior

Suzanne BeDell
Managing Director, Education Reference & Continuity Books

Philippe Terheggen
Managing Director, Science, Technology & Medical Journals



Researcher Academy On Campus

Certificate of Attendance

ELSEVIER

This certifies that

Deep Shikha Sharma

has attended the following

Two Day Webinar: Navigating the Pathways of Research Publishing in Scopus-indexed journals- Day 2

at Research and Consultancy Cell, Vidya Prabodhini College (VPCCECM), Goa,
on Saturday 25 September, 2021

Presented by Vishal Gupta Sr. Consultant, Elsevier, Dr Sumit Narula Deputy Dean Research, Amity University Gwalior

Suzanne BeDell
Managing Director, Education Reference & Continuity Books

Philippe Terheggen
Managing Director, Science, Technology & Medical Journals

The Indian Pharmaceutical Association-
Maharashtra State Branch's
BOMBAY COLLEGE OF PHARMACY

CERTIFICATE OF PARTICIPATION

THIS IS TO CERTIFY THAT

Deep Shikha Sharma

has attended and participated as **DELEGATE** in the
Half day Webinar on the theme
"Rise of Dermacosmeceutical Market as an upcoming Trend and Opportunities for Pharma Graduates"
held on **January 28th, 2021.**
Organised by Department of Pharmaceutics under the aegis of
Internal Quality Assurance Cell, Bombay College of Pharmacy.

PROF. KRISHNAPRIYA MOHANRAJ	**DR. UJWALA SHINDE**	**DR. HARITA DESAI**
Incharge Principal	**Convener**	**Organising Secretary**

Anton Paar

Deep Shikha Sharma

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

Rishi gupta

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

Society of Pharmaceutical Education & Research (SPER)

in association with

School of Pharmaceutical Sciences, LPU

organized

April 30, 2021

WEBINAR

Theme

Formulation Development of Nutraceutical / Herbal Products: Global regulation and Prospective Opportunities

CERTIFICATE OF PARTICIPATION

This is to certify that

DEEP SHIKHA SHARMA

has participated in the webinar as delegate.

DR. UPENDRA NAGAICH
Secretary,
Society of Pharmaceutical Education
& Research [SPER], India

DR. BIMLESH KUMAR
Assoc. Prof., School of Pharmaceutical Sciences,
Lovely Profesional University, India
President, SPER Punjab State Branch

AIPFC
ASSOCHAM IP Facilitation Centre

ASSOCHAM
Celebrating 101 Years

LOVELY PROFESSIONAL UNIVERSITY

CERTIFICATE OF PARTICIPATION

This is to certify that

DEEP SHIKHA SHARMA

has attended

IPR awareness webinar on

"PATENTING FOR ACADEMIC INSTITUTIONS"

Organized by, ASSOCHAM, Lovely Professional University, Phagwara, and IP Office

held on 23rd December 2021.

Mr. Mohd. Nahid Alam
Joint Director & Head,
ASSOCHAM IPR Council,
New Delhi.

Dr. Monica Gulati
Senior Dean & Head, Lovely School of
Pharmacy, Lovely Professional
University, Phagwara, Punjab.

Drug Discovery & Development Cluster
Jointly organized with
Amity Institute of Biotechnology | Amity University Chhattisgarh

AMITY UNIVERSITY

International e-Workshop
on "Computational Approaches in Drug Design & Therapeutics"

CERTIFICATE

This is to certify that Dr./Ms./Mr. **DEEP SHIKHA SHARMA** has participated in the **International e-Workshop** on **"Computational Approaches in Drug Design & Therapeutics"** held on February 20, 2021 organized by Drug Discovery & Development Cluster Jointly with Amity Institute of Biotechnology, Amity University Chhattisgarh. We appreciate his/her remarkable presence in the Workshop.

Dr. Puneet Gupta
Organizing Secretary &
Chief Coordinator
Drug Discovery & Development Cluster

Dr. Jaya Pandey
Joint Secretary &
Chief Coordinator
Drug Discovery & Development Cluster

Prof. (Dr.) B.C. Das
Dean - Health & Allied Sciences
Amity University Uttar Pradesh, Noida

Dr. W. Selvamurthy
President - ASTIF
Amity University Uttar Pradesh, Noida

DEPARTMENT OF PHARMACEUTICAL SCIENCES
MAHARSHI DAYANAND UNIVERSITY, ROHTAK

(A State University established under Haryana Act No. 25 of 1975)
(NAAC Accredited 'A+' Grade)

Two Days National Conference on

"Quality Control of Indian Medicinal Plants – Standardised Raw Material to Finished Products"

25-26th March 2021

Sponsored By:
University Grants Commission, New Delhi
under Special Assistance Programme-II

Certificate of Participation

This is to certify that Prof./Dr./Mr./Ms. **DEEP SHIKHA SHARMA**

from **LOVELY PROFESSIONAL UNIVERSITY** attended

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

Prof. Sanju Nanda	Prof. Munish Garg	Prof. Harish Dureja	Dr. Vineet Mittal	Dr. Saloni Kakkar
Patron	Convener	Co- Convener	Organising Secretary	Joint Organising Secretary

Certificate No. 225574

National Conference on
Recent Trends in Biomedical Sciences
(RTBS-2020)

2nd and 3rd July, 2021

Certificate of Participation

This is to certify that Dr. / Mr. / Ms. **Deep Shikha Sharma**

of **Lovely Professional University Punjab** presented Oral / Poster

on title **Preliminary Screening of Formulation Variables for Development of 5-Fluorouracil Loaded Nanostructured Lipid Carriers**

in the National Conference on " **Recent Trends in Biomedical Sciences - 2020** " organized by Department of Medical Laboratory Sciences, Lovely Professional University, Punjab.

Date of Issue : 19-07-2021
Place of Issue: Phagwara (India)

Prepared by
(Administrative Officer-Records)

Organizing Secretary

Monica Gulati

Chairman

14TH CHANDIGARH SCIENCE CONGRESS

CHASCON-2020

Certificate of Appreciation

This certificate is awarded to Mr./Mrs./Dr./Prof. ***Deepshika Sharma*** for Oral Presentation in 14th Chandigarh Science Congress jointly organised by Panjab University and Chandigarh Region Innovation and Knowledge Cluster on 17th-19th December, 2020.

Presentation entitled- ***Formulation of 5-Flurouracil loaded nanostructured lipid carriers for the management of diabetes retnopat***

Section- ***Pharmaceutical sciences***

Prof. Desh Deepak Singh	Prof. Ravinder Kumar Singla	Prof. Sandeep Sehajpal
Coordinator	DUI	Co-coordinator

SWAMI VIVEKANAND GROUP OF INSTITUTES

WOMEN FORUM

SVCP
NURTURING HEALTH

INTERNATIONAL CONFERENCE

On

CURRENT SCENARIO IN ADVANCEMENTS OF PHARMACEUTICAL EDUCATION & RESEARCH: CHALLENGES & PROSPECTS

SVCPCON-2020

29th-31st December 2020

Organized by

SWAMI VIVEKANAND COLLEGE OF PHARMACY, BANUR, PUNJAB

Certificate of Participation

This is to certify that Dr./Mr./Ms. Deep Shikha Sharma of Lovely Professional University participated as Delegate during the conference held on 29th-31st December 2020

.

Ms. Sonia Pahuja	*Dr. Prerna Sarup*	*Mr. Ashok Garg*	*Mr. Ashwani Garg*
Organising Secretary	Conference Chair	President	Chairman

Virtual Conference on

"Pharma Vision 2k25"

Organised by Shri Balaji Book Distributors

&

Supported by Association of Pharmaceutical Teachers of India (APTI)

Proudly presented

CERTIFICATE OF PARTICIPATION

to

Deep Shikha Sharma

For attending one day **National Virtual Conference** on **"Pharma Vision 2k25"** held on **Sunday, 27th September 2020.**

Sd/-	Sd/-	Sd/-	Sd/-	Sd/-
Dr. Shailendra Saraf	Shri Atul Nasa	Dr. Raman Dang	Dr. Abhishek Tiwari	Mr. Amit Jha
Chief Guest	Guest of Honor	Secretary APTI	Organizing Secretary	Co-ordinator

RAMANBHAI PATEL COLLEGE OF PHARMACY

CHARUSAT
CHAROTAR UNIVERSITY OF SCIENCE AND TECHNOLOGY
Accredited "Grade A" by NAAC, Government of India
www. charusat.ac.in

Certificate

This is to certify that

Ms. DEEP SHIKHA SHARMA

has attended Webinar on "Application of Basic statistics and Design of Experiments tools in Pharmaceutical Research"

organized by Ramanbhai Patel College of Pharmacy, Charotar University of Science and Technology, Changa during

(September 3-5, 2020)

Dr. Gayatri Patel
Coordinator - RPCP

Dr. Manan Raval
Principal - RPCP

Certificate

CHITKARA COLLEGE OF PHARMACY
CHITKARA UNIVERSITY, PUNJAB

6th International Conference on
"Pharmacy Practice Clinical Research and Pharmacovigilance Trends-2019" (PPCRPV-2019)"

THIS IS TO CERTIFY THAT

Dr./ Mr./Ms. Deep Shikha Sharma

OF

Lovely Professional University

has participated in the International Conference

"PPCRPV-2019"

on 8th and 9th November, 2019

as Student/Research Scholar/Faculty Participant

Dr. MADHU CHITKARA
CHIEF PATRON
Vice Chancellor | Chitkara University

Dr. SANDEEP ARORA
CHAIRMAN & CONVENER
PPCRPV-2019 |Chitkara University

Dr. THAKUR GURJEET SINGH
ORGANISING SECRETARY
PPCRPV-2019 | Chitkara University

Dr. MANJINDER SINGH
ORGANISING SECRETARY
PPCRPV-2019 | Chitkara University

Dr. Kishore Gnana Sam
Education Partner
Gulf Medical University

Dr Javed Aali
Indian Pharmaceutical Association

IAMBSSCON 2019

OCTOBER 19, 2019 | PGIMER CHANDIGARH

Organized by:

Integrated Association of Medical, Basic and Social Scientists (IAMBSS)

Certify that

Deep Shikha Sharma

has participated as Delegate/~~Speaker~~/~~Organizer~~/ ~~Chaired~~ a session in

IAMBSSCON 2019, PGIMER , Chandigarh

1st National Conference of IAMBSS (IAMBSSCON-2019) is accredited by Punjab Medical Council for Continuing Medical Education (CME) to provide Four (4) Credit Hours for Conference

Dr Ujjawal	***Dr Jitender Gairolla***	***Mr Rajeev K Chaudhary***	***Dr. Vijay Kumar***
Organising Chairman, IAMBSSCON -2019	*Organising Secretary, IAMBSSCON-2019*	*Co-Organising Secretary, IAMBSSCON-2019*	*Member, Punjab Medical Council*
President, IAMBSS	*General Secretary, IAMBSS*	*Council Member, IAMBSS*	

Observer
Punjab Medical Council

Certificates No. 179456

Certificate of Recognition

This is to certify that Prof./Dr./Mr./Ms. DEEP SHIKHA SHARMA has participated as **Local Organizing Committe Member in International Conference of Pharmacy (ICP-2019) on the theme of " Pharmacy: Realigning the focus on health"** held on 13-14th September 2019 organized by School of Pharmaceutical Sciences, Lovely Professional University, Punjab in a collabration with Indian Pharmacy Graduates' Assocaition (IPGA) Phagwara chapter.

Date of Issue: 18-10-2019
Place of Issue: Phagwara (India)

Prepared by
(Administrative Officer-Records)

Dr. Bimlesh Kumar
Organizing Secretary

Dr. Monica Gulati
Chairperson, Local Organizing Committee

NATIONAL SYMPOSIUM

on

INNOVATION AND ENTREPRENEURSHIP

An Initiative of UIPS Institution Innovation Council (IIC) under the aegis of Innovation Cell, MHRD, Govt. of India

organized by

University Institute of Pharmaceutical Sciences

Panjab University, Chandigarh – 160 014 India

April 9, 2019

Certificate of Participation

Presented to

Deep Shikha Sharma

for active participation as a ***Delegate***

Indu Pal Kaur

Professor Indu Pal Kaur
Organizing Secretary

Kanwaljit Chopra

Professor Kanwaljit Chopra
Symposium Chair & Chairperson, UIPS

CHITKARA UNIVERSITY

MMU MULLANA

ADESH GROUP OF INSTITUTIONS

Certificate

CHITKARA COLLEGE OF PHARMACY, CHITKARA UNIVERSITY, PUNJAB

PHARMACY PRACTICE SUMMIT-2019

Collaborative Summit

"Advanced Clinical and Community Pharmacy Services in Qualitative Therapeutics and Healthcare Delivery: The serious missing link"

This Is To Certify That ~~Mr.~~/Ms.

Deep Shikha Sharma

of

Lovely Professional University

has participated in the **PHARMACY PRACTICE SUMMIT-2019**

on 19th April to 20th April, 2019

as Student/Faculty Participant.

Dr. MADHU CHITKARA	**Dr. SANDEEP ARORA**	**Program Collaborators**	**Dr. THAKUR GURJEET SINGH**
PATRON	Convener & Chairman	PCI, IPA, APTI, IHPA	Organising Secretary
Vice Chancellor	PPS-2019	PPS-2019	PPS-2019
Chitkara University, Punjab	Chitkara University, Punjab	Chitkara University, Punjab	Chitkara University, Punjab

CHITKARA UNIVERSITY

NEELAM HOSPITAL
Superspeciality Health Care

Certificate

CHITKARA COLLEGE OF PHARMACY, CHITKARA UNIVERSITY, PUNJAB
&
Neelam Hospital, Rajpura & Rajindra Medical College & Hospital, Patiala

Pharmacy Practice Module-Advance learning series on Emerging Trends in Health Care: Clinical correlation and their outcome for Therapeutic Management-2019
***Module VII:* Advances in Internal Medicine-II: Pharmacotherapy of Chronic Disease**

This Is To Certify That Dr./~~Mr.~~/Ms.

Deep Shikha Sharma

of

Lovely Professional University

has participated in the Pharmacy Practice Module VII

"ETHC-2019"

on 18th to 21th April, 2019

as Expert Session/Chairman/Event Coordinators/Organizing Secretary/Technical Expert/Student/Faculty Participant

Dr. MADHU CHITKARA
PATRON
Vice Chancellor
Chitkara University, Punjab

Dr. SANDEEP ARORA
CONVENER & CHAIRMAN
ETHC-2019
Chitkara University, Punjab

Dr. THAKUR GURJEET SINGH
ORGANISING SECRETARY
ETHC-2019
Chitkara University, Punjab

MINISTRY OF AYUSH

6th EDITION 2018

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A JOURNEY TO HEALTHY WORLD

LOVELY PROFESSIONAL UNIVERSITY

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arogya sangashthi

CERTIFICATE OF RECOGNITION

This is to Certify that

Prof./Dr/Mr./Ms./Mrs. Deep Shikha Sharma

has participated as Speaker/Chairperson/Co-Chairperson/Moderator/Poster Evaluator/Member - LOC in Integrated Conference on Ayurveda, Agriculture, & Pharmaceutical Science held on 13th & 14th october 2018 in Lovely Professional University, Punjab.

V. Sharma
Shri Vijay Sharma
Chairman, Namo Gange Trust

Monica Gulati
Dr. Monica Gulati
Chairman, Organizing Committee

Dr. Manish Vyas
Organising Secretary

NAMO GANGE TRUST, 12/52, Site-II, Loni Road Industrial Area, Mohan Nagar, Ghaziabad 201007

Toll Free: 1800 3000 0639, Web: www.namogange.org

Supported by | Aviral Ganga One Step for Clean Ganga | Yogshala Jobs.com | Knowledge Partner APSECR

Publication Partner | āyurved Sutrā | YOGIC HERALD

Deep Shikha Sharma

LPU School of Pharmaceutical Sciences, Punjab

attended a session on

Basics of Rheology

Date: August 03, 2018

Time: 09:30 a.m. to 17:00 p.m.

Venue: Anton Paar India's Application Lab, Gurugram

Contents

The training provided the essential knowledge for:

- Basic rheological terms and measurement variables
- Flow behavior of viscous materials and deformation behavior of viscoelastic materials
- Basic test methods
- Interpretation of measuring diagrams
- Hands-on-session on Rotational and Oscillatory mode of Rheometer along with data interpretation

Date of issue: August 03, 2018

3/8/18

Deepika Malpani
Application Specialist, Characterisation
Anton Paar India Pvt. Ltd.

LOVELY PROFESSIONAL UNIVERSITY

Qsutra®
Academy For Excellence

CERTIFICATE OF PARTICIPATION

This is to certify that Prof. / Dr. / Mr. / ~~Ms.~~ Deep Shikha Sharma has participated as a **Delegate** / **Resource person** in Conference on "**Design of Experiments**" jointly organized by **Qsutra** & **School of Pharmaceutical Sciences, Lovely Professional University,** Punjab on 13th - 14th July 2018 .

Monika Gulati
Prof. Monika Gulati
Sr. Dean - LFAMS, LPU

Qsutra Academy

Mr. Madhu Madhavan
Founder & CEO – Qsutra

Dr. Saurabh Satija
Organizing Secretary

LOVELY PROFESSIONAL UNIVERSITY

PHYTOCON-2018

SPRINGER NATURE

Certificate of Participation

"Author Workshop jointly organized

by

Lovely Professional University, Punjab and Springer Nature"

This is to certify that

Dr./Mr./Ms./ Prof. Deep Shikha Sharma

has successfully participated in Author Workshop

on "How to Write and Publish Scientific Articles and Manuscripts"

on 15th April, 2018 at Lovely Professional University, Punjab.

Monica Gulati

Prof. Monica Gulati
Sr. Dean, Lovely Faculty of Applied Medical Sciences (LFAMS)
Lovely Professional University

Sanjiv Goswami
Managing Director
Springer (India) Pvt. Ltd.

Certificate No. 92166

LOVELY PROFESSIONAL UNIVERSITY

Transforming Education Transforming India

PHYTOCON-2018
Saturday, April 14, 2018
LPU, Punjab, India

Certificate of Participation

This is to certify that Prof./Dr./Mr./Ms. **Deepshikha Sharma**

has participated as **Speaker / Chairperson / Co-Chairperson / Moderator / E-Poster Evaluator / Oral Presentation Evaluator / Member LOC** *in the* **PHYTOCON 2018** *- International Conference on* **"Commercialization of Medicinal Plant Products : Lab Techniques to Trade"** *held on April 14, 2018 organized by School of Pharmaceutical Sciences, Lovely Professional University, Punjab.*

Date of Issue : 14-04-2018
Place of Issue: Phagwara (India)

Prepared by
(Administrative Officer-Records)

Dr. Navneet Khurana
Chairman, Scientific Services

Dr. Saurabh Satija
Organizing Secretary

Prof. Monica Gulati
Chairman - LOC

Chairman - LOC

HUMAN RESOURCE DEVELOPMENT CENTER

[Under the Aegis of Lovely Professional University, Jalandhar-Delhi G.T. Road, Phagwara (Punjab)]

Certificate No. 73568

Certificate of Participation

This is to certify that **Ms. Deep Shikha Sharma** D/o Sh. Tarsem Lal Sharma, Assistant Professor, Department of Pharmaceutical Sciences, Lovely Professional University, Phagwara participated in **Workshop on Role of HPLC and Analytical Method Development in Dosage Form Design** organized by Human Resource Development Center, Lovely Professional University from **February 02, 2018** to **February 03, 2018** and obtained **'B'** Grade.

Prepared by
(Administrative Officer –Records)

Head
Division of Human Resource

Head
Human Resource Development Center

Date of Issue: 03-02-2018
Place: Phagwara (Punjab)