Development and Evaluation of Simvastatin Loaded Transfersomal Hydrogel for the Management of Atherosclerosis

M.Phil Thesis

by

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Development and Evaluation of Simvastatin Loaded Transfersomal Hydrogel for the Management of Atherosclerosis

Thesis submitted by

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AUTHOR'S DECLARATION

I, Syeda Ayesha Mazhar, hereby declare that the thesis titled **"Development and Evaluation of Simvastatin Loaded Transfersomal Hydrogel for the Management of Atherosclerosis"** submitted to the Department of Pharmacy, Faculty of Biological Sciences, Quaid-i-Azam University, Islamabad for the award of degree of Master of Philosophy in Pharmacy (Pharmaceutics) is the result of research work carried out by me under the supervision of Dr. Kifayat ullah Shah during the period 2021-2023. I further declare that the results presented in this thesis have not been submitted for the award of any other degree from this University or anywhere else in the country/ world and the University has the right to withdraw my MPhil. degree if my statement is found incorrect any time even after my graduation.

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This thesis is dedicated to my beloved parents, thank you for your endless love, support, pray, guidance and everything you have done. I am thankful to my husband Hafiz Muhammad Musharaf for supporting me during my research work and being integral part of my motivation towards research.

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Syeda Ayesha Mazhar

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List of Abbreviations

Abbreviations	Description
3D	Three Dimension
Abs	Absorbance
BCS	Biopharmaceutics Classification System
Conc	Concentration
DF	Dilution Factor
DL	Drug Loading
DLS	Dynamic Light Scattering
DSC	Differential Scanning Calorimetry
EE	Encapsulation Efficiency
FTIR	Fourier Transform Infrared Spectroscopy
Hrs	Hours
HDL-c	High Density Lipoproteins cholesterol
ICH	International Council for Harmonization
LC	Loading Capacity
M	Molarity
m^2	Meter square
mg	Milli-gram
Mkd	Marketed
ml	Mille-liter
nm	Nanometers
NPs	Nanoparticles
PS	Particle Size
RPM	Revolutions Per Minute
SC	Stratum Corneum
TG	Triglycerides
TC	Total Cholesterol
UV	Ultraviolet
vLDL	very Low-Density Lipoproteins
XRD	X-ray Diffraction

Abstract

Simvastatin is mainly used for management of atherosclerosis, possess problems when administered orally like poor solubility (BCS class II) and low bioavailability (<5%). The current study aimed to design and developed simvastatin loaded transfersomes based transdermal hydrogel for the targeted delivery. The formulation was evaluated in Poloxamer-407 induced hyperlipidemia rat model for the management of atherosclerosis. Thin film hydration method was used for the preparation of simvastatin loaded transfersomes which was loaded into the carbopol 934 hydrogel. Simvastatin loaded transfersomes were evaluated through using particle size analysis, zeta potential, PDI, DSC, FTIR, XRD, in vitro drug release, ex vivo, skin irritation studies and in vivo studies. The transfersomes revealed uniform particle size of 128.7 nm, PDI 0.273 and zeta potential of -20.4 mV and EE of 83.1%. In vitro drug release showed sustained release of simvastatin. *Ex vivo* permeation studies showed maximum permeation through skin at pH 7.4. *In vivo* studies revealed remarkable decrease in elevated serum lipids compared to that of untreated and free simvastatin treated rat model. The poloxamer injection considerably elevated the levels of cholesterol in all groups during the first 24 hours. Similar was the case with vLDL, the level of HDL decreases sharply within first 24 hours after the poloxamer 407 injection. The normal group didn't show any significant change in the levels of cholesterol, TG, HDL, and vLDL which proves the hyperlipidemic action of poloxamer 407. The transdermal administration of free simvastatin and simvastatin loaded transfersomal hydrogel decreased the level of hyperlipidemia that also decreased the atherogenic index in 72 hours study. Comparing the atherogenic index between the diseased control group, free simvastatin hydrogel treated group and simvastatin loaded transfersomal hydrogel treated-group, it is shown in results above that the atherogenic index was lowered in simvastatin loaded transfersomal hydrogel treated group of Poloxamer 407 induced hyperlipidemia. Increased cholesterol level and triglyceride level with decreased high-density lipoproteins in serum may give rise to atherosclerotic plaque. Simvastatin loaded transfersomal hydrogel reduced the TG and TC levels significantly. LDL levels were significantly decreased by simvastatin loaded transfersomal hydrogel therefore results imply that simvastatin loaded transfersomal hydrogel provides advantage by decreasing lipid profile. So, simvastatin loaded transfersomes based transdermal hydrogel showed great potential for management of atherosclerosis.

CHAPTER 1

INTRODUCTION

1. INTRODUCTION

1.1. Atherosclerosis

Atherosclerosis underlies the pathogenesis of coronary (Pothineni *et al*., 2017); cerebral and peripheral arterial diseases (Basatemur *et al*., 2019) and is the cause of morbidity and mortality (Geovanini and Libby, 2018). The arteries affected in atherosclerosis are the medium sized as well as large sized (Katakami 2018) due to fat deposition (Nowak *et al*., 2017, Geovanini and Libby, 2018). Atherosclerotic plaque comprises of cells (smooth muscle cells, macrophages, T cells etc.) (Yang *et al*., 2020), extracellular matrix (collagen, elastic fibers, proteoglycans), lipids (intracellular, extracellular) (Kattoor *et al.,* 2017), with calcification in later stages (Xu *et al.,* 2019). It is an intimal thickening and fat accumulation (Shah 2019), raised lesions that vary in size (Jebari *et al.,* 2022). At first fatty streaks are forced which are composed primarily of lipid forming macrophages which evolve into the plaque in later stages.

1.1.1. Causes

Arteries carry blood away from the heart and are covered by epithelium for smooth shape and help in blood flow and this is the point from where Atherosclerosis starts, damage in endothelium causes it.

Common causes include:

- Increased levels of LDL
- Elevated inflammation and blood pressure
- Obesity
- Smoking

Damage in endothelium leads to formation of plaque in the walls of large and medium sized arteries. These plaques then can be matured and increases the chances of blockage which may result in heart stroke, heart attack or other health problems. These symptoms occur mostly after middle age until narrowing becomes severe and causes pain blockage can rupture suddenly as well.

1.1.2. Risk factors

These symptoms occur mostly after the middle age until narrowing becomes severe and causes pain, blockage can rupture suddenly as well.

Almost 90 % of all the cardiac attacks are due to this. It includes,

- obesity
- Diabetes
- High consumption of alcohol
- Elevated blood pressure
- Increased cholesterol
- Lack of healthy diet
- No exercise
- Smoking
- Stress

1.1.3. Diagnosis

First, physical exam is taken, and some other tests are:

- Angiogram
- Ankle-brachial index
- Blood and serum tests
- MRA or CT scan
- EKG

1.2. Historical Background

Since the 19th century, scientists have been captivated by the multilamellar texture of skin. Homalle (1853) and Duriau (1856) were the first to realize that dermal surface, notably structure of the skin dermis or epidermis, exhibited permeability and existence of barrier between epidermis and SC. New visualization and characterization (XRD, DSC, FTIR) techniques are being introduced in recent decades, have a wide range of applications in the research of the barrier properties of various materials as well (Potts *et al.,* 1985).

1.2.1. Structure of the SC

Simple two-part structural arrangement, with corneocytes submerged in a lipid matrix, as shown in frozen sections swelled in basic buffer and stained with a dye. "Brick and mortar system" explains this effectively (Michaels *et al.,* 1975).

The stratum corneum’s structural components are:

a) Corneocytes
b) Corneodesmosomes
c) The mortar lipids that line the winding passage in between stacked corneocytes

d) Lipolytic & proteolytic enzymes in a battery.
e) Secreted constituents of the epidermal lamellar bodies just at stratum corneum-stratum granulosum contact.

A SC acts as a barrier to transdermal medications transport, requires all these components. The barrier can be weakened by interfering with or changing the functioning qualities of any of these components.

1.3. Simvastatin

Simvastatin is a white, non-hygroscopic, crystalline powder which has molecular weight of 418 g/mol and half-life of about 1 to 2 hours. According to biopharmaceutics classification system (BCS) class II i.e., simvastatin has low solubility and high permeability. When orally administered, simvastatin shows oral bioavailabity of less than 5 %. It is one of the effective lipids lowering medication which mainly act by reducing the levels of low-density lipoproteins levels up to 50 %. Marketed product of simvastatin is Zocor (brand-name). It belongs to the statin drug class, which lowers the risk of cardiovascular disease and regulates elevated cholesterol levels by blocking the liver's natural synthesis of cholesterol. One of the first line strategy to overcome the fatty streak formation is the use of simvastatin (Barale *et al.,* 2018)(Talreja *et al.,* 2018). The hydroxymethyl glutaryl-coenzyme A (HMG-CoA) reductase, which catalyzes the hydrolysis of HMG-CoA to mevalonic acid (Balasubramanian and Maideen, 2021) and is the third step in a series of metabolic reactions involved in the production of several biomolecules involved in lipid metabolism and transport, including cholesterol and low density lipoprotein (LDL), which is sometimes referred to as bad cholesterol, is specifically competitively inhibited by statin drugs (Fytsilis *et al.,* 2022).

1.3.1. Mechanism of action

Simvastatin is a prodrug, which converts into the active metabolite dihydroxy acid which is similar to the HMG-CoA reductase enzyme and competitively binding to the HMG CoA enzyme receptor as shown in Figure 1.1 (Fytsilis *et al.,* 2022) and inhibit the conversion to mevalonate which will later convert into the cholesterol. This mechanism of simvastatin occurs in the liver hence help in decrease of cholesterol in liver and decreases the concentration vLDL and LDL.

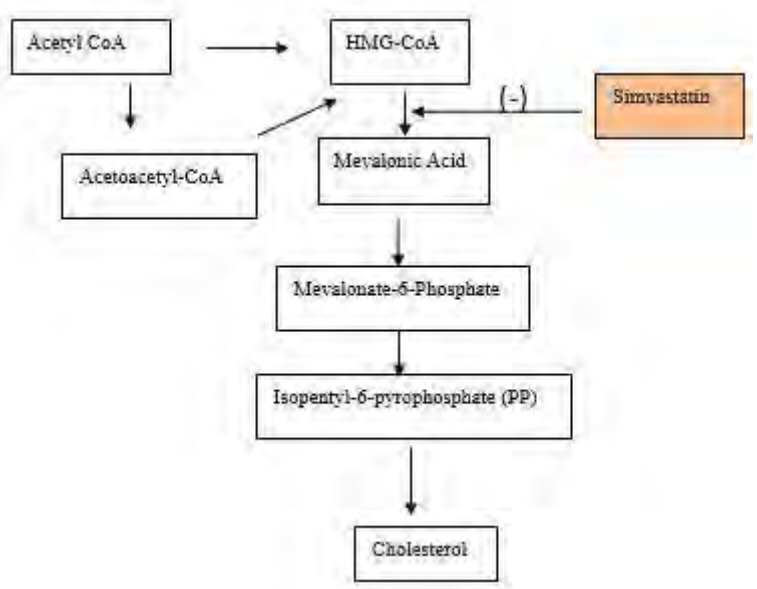

Figure 1.1. Mechanism of action of simvastatin.

1.4. Transdermal Drug Delivery

Most of the medications, which were given orally in about 74% of cases, were unsuccessful. To solve these issues, the transdermal medication delivery system was developed. In contrast to topical drug administration, transdermal drug delivery entails administering the medication via the skin to have a systemic impact. In contrast to topical drug administration, transdermal drug delivery entails sending a chemical to the reproductive system through the skin. Systemic effects of medication delivery through the skin. Compared to conventional oral and invasive methods, topical medication administration provides several benefits. Reduced first-pass hepatic metabolism, increased therapeutic efficacy, and maintenance of stable plasma drug levels are a few benefits of transdermal drug administration. Transdermal drug delivery (TDDS) is the process of delivering medications to the epidermal and/or dermal regions of the skin for local therapeutic impact only while a significant proportion of the drug is delivered in the systemic bloodstream. The identification of elements causing impermeability has allowed for the routine use of the skin as a medication delivery system. Through the skin, medications can be absorbed four times. Micro sealed system Gradient charged system The membrane permeation-controlled system Matrix diffusion system.

1.4.1. The benefits of using a transdermal medication delivery system (TDDS)

Through TDDS, patients can administer medications with a controlled release. The first-pass impacts might be lessened by such consequences.

- Reduce the detrimental effects on the entire body.
- Increasing the effectiveness of the dose will result in higher patient compliance as well as more constant medication blood levels during treatment.
- The optimum method for medications with short half-lives and low therapeutic indices, TDDS lowers blood levels of drug variations.
- Lowering the drug's plasma levels can lessen side effects.
- It is simple to stop taking the drug in cases of toxicity.
- To increase compliance and decrease dose frequency, use patience.
- There are negative side effects since TDDS delivers the medication gradually over time. Treatment failure and irregular dose can both be prevented.
- TDDS makes it simple to avoid side effects associated with GIT and decreased intestinal absorption of medications.
- When compared to the oral dose of this medication, a lower daily dose can still have the therapeutic benefit.
- Patient compliance has improved, and there is less variation between and among patients.

1.5. Why Nanoparticles

For a variety of reasons, such as primary hepatic metabolism, unpleasant side effects, unwillingness to undergo invasive procedures, and poor patient adherence, effective and successful treatment may not always be possible. Due to several factors, such as first-pass hepatic metabolism, negative effects, the invasive nature of the treatment, and low patient compliance, an effective and successful treatment regimen is uncommonly accomplished. For the issue mentioned above, liposomes and other lipid- based vesicles have been used (Elsayed *et al.,* 2007). The lipid matrix of the film restricts absorption on the dermis surface and stops the active ingredient's continuous transfer from the nanocarriers into the skin, increasing the active ingredient's distribution in the dermis and phospholipid transfer between the layers. Drug diffusion over the skin is made possible by liposomes in three different ways: through the plasma membrane, lipid nanoparticles, and lipid nanoparticles. Liposomes are therefore used as the main carrier for cutaneous medicine delivery as opposed

to transdermal drug administration. Conventional liposomes have flaws, including B. Poor hydrophilic drug screening, a brittle membrane that causes leaky behavior, and rapid elimination. He introduced transfers, a fresh kind of mechanism, in the 1990s. Because transfersomes are extremely adaptable carriers, because they are formed of phospholipids, mineral activator (EA), and substances that smooth the membrane, transfersomes are remarkably flexible transporters (such as Tween 80, Span 80, and sodium cholate). The membranes of the grafts grow more pliable as they reach the opening of the skin, allowing passage through the pores. This is referred to as "self- deformability optimization".

1.5.1. Role of nanocarriers in the treatment of atherosclerosis

When taken in form of nanoparticles, simvastatin increased absorption, half-life, and mean residence time (Anwer *et al.,* 2019). As prospective nanocarrier-based formulations for the treatment of atherosclerosis, various nanocarrier formulations have been investigated which include liposomes, SLNs, NLCs, polymeric micelles, polymeric NPs, nano-capsules, ethosomes, and lyotropic liquid crystalline nanoparticles but all of them have one or more limitation in its use.

1.5.2. Transfersomes

In 1990, transferosomes were introduced and were considered a new kind of the technology. Transfersomes are made of phospholipids and edge activator (EA), a membrane-softening compound (such as tween 80, span 80, and sodium cholate) that enables transfersomes to be extremely flexible. Transfersomes can travel through skin pores because when they penetrate the skin, their membrane becomes more flexible and hence helps to penetrate easily. This phenomenon is known as Self- optimizing deformation (Abd El-Alim *et al.,* 2019). The transfersomes have high permeation ability (Ghasemiyeh and Mohammadi-Samani, 2020) and also incredibly flexible, allowing them to effortlessly flow through even the smallest pores. Ethosomes are nanoparticles made of phospholipids, water, and ethanol in quantities ranging from 20 to 50 %. Because ethosomes contain high amounts of ethanol, they alter the skin lipid bilayer, which enhances vesicles capacity to enter the stratum corneum. Phosphatides, glycosides, and proteins are all present in invadesomes, which are nanoparticles and are used for treatment of different diseases. Most of the time, factors such as primary hepatic metabolism, unfavorable side effects, the

unwillingness to use invasive therapies, and poor patient compliance make effective and successful therapy impossible. To address these problems, a variety of drug delivery methods are being designed, and research from the past ten years suggests that transdermal delivery systems are a promising approach because they are intrusive and have brief initial effects. Therefore, it is necessary to address the blocking property of the skin that limits or reduces the drugs transdermal transit. The drawback of traditional lipid nanoparticles is that they does not circulate throughout the body. Therefore, rather than transdermal medication administration, liposomes are typically used as carriers for cutaneous drug delivery. Traditional lipid nanoparticle drawbacks include slow elimination, low hydrophilicity, active ingredient incorporation, and membrane penetration behavior.

1.5.2.1. Structure of transfersomes

A lipid bilayer, surfactants or edge activators and at least one internal aqueous chamber are all features of transfersomes, which are vesicular carrier systems. Ultra-deformable vesicles with the ability to self-optimize and regulate themselves are produced when this hydrophilic core is coated with a lipid bilayer. Due to their elastomeric nature, transfersomes can be deformed and compressed into intact vesicles without suffering serious harm from skin constrictions or pores that are much smaller than the vesicles (Sivannarayana *et al.,* 2012).

As a straightforward EA, transfersomes, a component of the phospholipid vesicle system, can be coupled with a single-chain surfactant. The deformability of vesicle membranes is enhanced by edge activators (EAs), which weaken membranes. They give a perfect balance that renders transfersomes flexible and ultra-flexible, resulting in better penetration capabilities, when paired in the ideal proportion with the right lipid (Lei *et al.,* 2013).

1.5.2.2. Advantages of transfersomes

- By passing through pores that are much smaller than their own diameters and maintaining their dimensions despite fragmentation after passing through the smaller pores, transfersomes address the fundamental problems with regular liposomes.
- Even after passing through the smaller pores, transfersomes remain intact

despite disintegration. When compared to conventional liposomes, the performance of transfersomes prepared using EAs has improved.

- ➢ Transferosome carriers are unique drug delivery systems that may deliver therapeutic medications with a wide range of solubility since they are made from hydrophilic and hydrophobic molecules and hence provide improved solubility and bioavailability profile (Sapkota and Dash, 2021).
- ➢ Due to their extreme deformability and elasticity, transfersomes can force their way past skin barrier obstructions that are up to ten times smaller than the diameter of a vesicle.
- ➢ High vesicle deformability can be used for topical and systemic treatments because it enables drug delivery across the skin without significantly reducing the number of vesicles and have sustained drug delivery (Anggraini *et al.,* 2017: Rane and Gujarathi, 2017).
- ➢ Regardless of their size, structure, molecular weight, or polarity, transferosomes are incredibly flexible and efficient at absorbing a variety of substances (Moawad *et al.,* 2017).

1.5.2.3. Limitations of transfersomes

Transfersomes are regarded as chemically unstable because of their propensity for oxidative destruction. Oxidation transfers may be somewhat minimized if such an aqueous media is desaturated before being purged with inert gases like nitrogen and argon. Spray drying as well as a post-drying manufacturing process can increase the stability of stored transferosomes. The difficulty of locating phospholipids in natural purity is another barrier to the utilization of transferosomes as a medication delivery system. As an alternative, synthetic phospholipids can be employed. The price of transpersonal products is influenced by the pricey machinery needed for manufacture as well as the raw materials utilized to get the lipids. Because of this, phosphatidylcholine is the most often utilized component of lipids.

1.6. Problem Statement

It belongs to BCS class II i.e., it has low solubility, and its half-life is about 1 to 2 hours. It shows oral bioavailability of less than 5 % due to extensive first pass metabolism. Transfersomes incorporating simvastatin carried by alternative transdermal route will increase solubility profile.

1.7. Aim and Objectives

1.7.1. Aim

Preparation and characterization of simvastatin loaded transfersomes based transdermal hydrogel and its evaluation against atherosclerosis.

1.7.2. Objectives

- Preparation of simvastatin loaded transfersomes.
- Evaluation of prepared transfersomes
- Incorporation of simvastatin loaded transfersomes into carbopol based hydrogel.
- Evaluation of simvastatin loaded transfersomal hydrogel.
- Investigation of simvastatin loaded transfersomal hydrogel in terms of in *vitro*, *ex vivo,* and *in vivo* studies.

CHAPTER 2

MATERIALS AND METHODS

2. MATERIALS AND METHODS

2.1. Materials

2.1.1. Chemicals and reagents

Simvastatin was purchased from Sigma Aldrich, Germany, phospholipid (phospholipon 90G), tween 80 and methanol. Hydrochloric acid was purchased from Riedel- De-haen, RDH, Labor Chemkalen, Gmbh & Co. Sodium di-hydrogen phosphate, dipotassium hydrogen phosphate, $NaBH_4$, sodium chloride were purchased from Merck, Scharlu, Hohenbrunn, Germany. Carbopol 934, TEA (Triethanolamine), chloroform, poloxamer 407, eucalyptus oil and distilled water were obtained from pharmaceutics lab (Department of Pharmacy, QAU, Islamabad).

2.1.2. Glass wares and instruments

2.1.2.1. Glass wares

Glass vials, spatula, beakers, eppendorf tubes, falcon tubes, micro-pipette, syringes 3cc, syringes 10cc, butter paper, gloves, tissue paper, magnetic stirrer, dialysis membrane with molecular weight cut–off 12-14kDA (Membrane filtration products, Texas, USA).

2.1.2.2. Instruments

Vacuum rotary evaporator, zeta sizer (ZS90, Malvern Instrument, Worstershine UK Multi), Magnetic Stirrer, Franz diffusion cell, Hot plat, pH meter (caliberated), Weighing balance, Centrifuge (Hemolabor technic Z-216 MK, Germany), Forrier Transform Infrared spectrophotometer, Bath sonicator, DSC, XRD, UV Spectrophotometer (HALO DB-20, UV-visible double beam spectrophotometry).

2.2. Methods of Preparation

2.2.1. Preparation of solutions

2.2.1.1. Phosphate buffer saline (PBS) pH 7.4

For preparation of buffer saline of pH 7.4, take 1000 ml of distilled water in beaker and add NaCl 800mg, KH_2PO_4 190 mg and Na_2HPO_4 2830 mg. Mix and stir well with help of magnetic and measure pH with help of pH meter (calibrated).

2.2.1.2. Phosphate buffer (PBS) pH 5.5

To make pH buffer 5.5, dissolve 6.8g KH_2PO_4 in 1000ml of distilled water and mix with help of magnetic stirrer then adjust pH up to 5.5.

2.2.2. Preparation of transferosomes

Simvastatin transfersomal vesicles were formed using a slightly modified thin film hydration process. Simvastatin was weighed and added in a 3:1 ratio to the combination of methanol and chloroform respectively. Then, until a thin film had developed at the bottom of the flask, this solution was added to the rotary flask evaporator at 100 rpm while it was held at 40 °C±1 °C. To create a transfersomal formulation, the film that had formed on the bottom of the flask was hydrated with phosphate buffer (pH 7.4) at 100 rpm for a further hour at 60 °C±1 °C without vacuum. The resulting formulation was run through 0.45 µm and 0.22 µm polyvinylidene fluoride (PVDF) syringe filters to remove big aggregates and free simvastatin from simvastatin transfersomes as shown in Figure 2.1. It was then kept for further analysis.

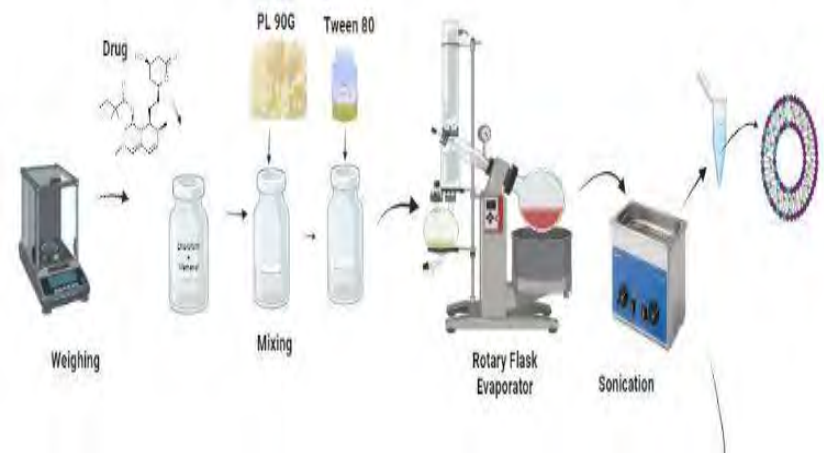

Figure 2.1. Schematic representation of preparation of simvastatin loaded transfersomes.

2.3. Optimization of Simvastatin Loaded Transferosomes

Simvastatin loaded transfersomes were optimized via design expert version 12 by Box Behnken model. Simvastatin, Phospholipon 90G, and Tween 80 concentrations were changed, and the effects of these changes on particle size, zeta potential, poly dispersity index, and % entrapment efficiency were noted.

2.4. Preparation of Standard Curves

Simvastatin transfersomes standard curve was prepared in methanol with the help of

UV spectrophotometry at 280 nm and the linearity range was found to be between 1-0.2 ug/ml.

2.5. Characterization of Transferosomes

2.5.1. Vesicle size (VS), poly dispersity index (PDI), zeta potential (ZP), and entrapment efficiency

Simvastatin transfersomes were visualized under Zetasizer (Malven Instrument, Worcestersire, UK) for their vesicle size, polydispersity index, and zeta potential. For this, blank formulation having no simvastatin and transfersomal formulation having simvastatin were evaluated using Zetasizer (Mann et al. 2009). 10 µl sample of fresh formulation was diluted by distilled water up to 100µl and was placed in Zetasizer for analysis.

Entrapment efficiency of simvastatin loaded transfersomes was determined by indirect method by taking supernatant and free drug concentration is measured. 1 ml of formulation was taken in eppendroph tube and centrifuged at 15000 rpm for 45 minutes at 4°C (Ahad *et al.,* 2018) (Tawfeek *et al*., 2020). The clear supernatant was separated and diluted with 1:10 with distilled water and analyzed on UV spectrophotometer to check its absorbance.

Following formula was used to determine %EE:

$$EE(\%) = Wt - wf\ x\ 100/Wf$$

where
Wt = total drug concentration,
Wf = concentration of free drug in supernatant.

2.5.2. Differential scanning calorimetry (DSC)

DSC was performed to analyze the thermal properties of drug that is enclosed in transfersomes i.e., simvastatin. Free drug simvastatin, freeze-dried powder of formulation and phospholipid thermal properties are identified from this technique (Pandya *et al.,* 2011) (Sariisik *et al.,* 2019).

2.5.3. X-ray diffraction study

Free simvastatin and a freeze-dried sample of transferosomes formulation were analyzed using X-ray diffraction (Fattahi *et al.,* 2016) (Heilmann *et al.,* 2021). The sample was placed in the sample holder, and the parameters were set according to the requirements, i.e., the current range was set at around 30mV, and the sample was

scanned at about 2θ range angles between 1 and 90 degrees.

2.5.4. Fourier transform infrared spectroscopy (FTIR)

The compatibility of transfersomes ingredients (free simvastatin and excipients) were investigated using FTIR (Garip *et al.,* 2010) (Vakhariya *et al.,* 2017). The sample, which included free simvastatin, phospholipids, and freeze-dried transferosomes, was treated with KBr solution before being analyzed in FTIR.

2.6. Preparation of Carbopol (934) Hydrogel

The transdermal gel formulation was prepared. For this purpose, optimized simvastatin transfersomes loaded into the Carbopol 934 hydrogel (Ubaid *et al.,* 2016) (Gaikwad *et al.,* 2012). 100 mg of Carbopol 934 was mixed in 10 ml of slowly stirring distilled water to make 1% gel. Finally, 2-3 drops of triethanolamine were added to adjust the pH of the gel. Proper care was taken to avoid the formation of non-dispersible lumps as shown in figure 2.2 (Sahni *et al.,* 2011).

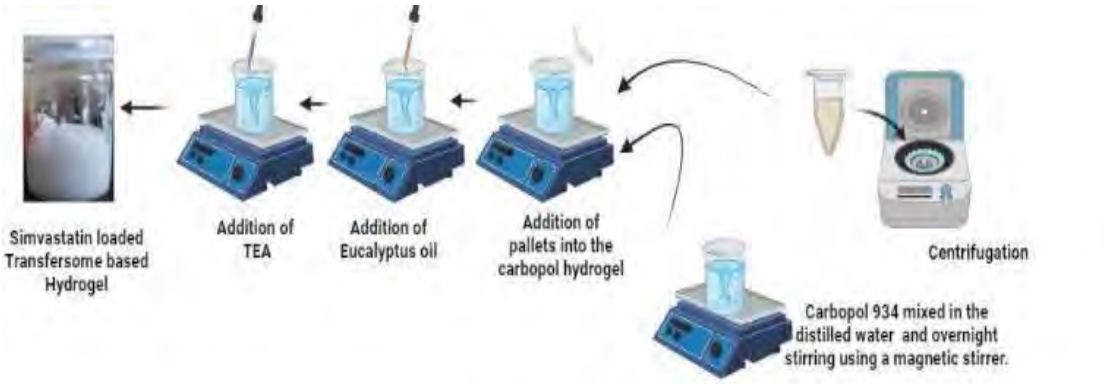

Figure 2.2. Preparation of simvastatin based carbopol hydrogel.

2.7. Characterization of Carbopol Based Hydrogel:

2.7.1. Physical appearance

Organoleptic approach of hydrogel was used to determine and ensure that the simvastatin loaded transfersomal hydrogel meets the criteria or requirements of hydrogel (Nilforoushzadeh *et al.,* 2021) (Abdelmalak and El-Menshawe 2012).

2.7.2. pH determination of hydrogel system

pH of hydrogels was determined by using pH meter (caliberated). 1% simvastatin loaded transfersomal hydrogel was prepared which was then analyzed through pH meter (calibrated) (Zhang *et al.* 2020) (Yuan *et al.,* 2022).

2.7.3. Drug content of hydrogel

The drug content was tested by dissolving 1g of gel in 100ml of phosphate buffer saline (PBS7.4) and shaking the solution for 2 hours until it homogenized properly (Tawfeek *et al.,* 2020) (Farghaly Aly *et al.,* 2019) . The resulting solution was then filtered out, and the drug concentration was determined using UV spectrophotometry at 280 nm. As a blank, PBS 7.4 was used (Arpa *et al.,* 2020). Equation used to obtain percentage drug content is as follows:

$$Drug\ content\ \% = \frac{Observed\ amount\ of\ drug\ in\ the\ formulation}{theoretical\ \ amount\ of\ drug\ in\ the\ formulation}\ x\ 100$$

2.7.4. Rheological studies

A digital rheometer is used to conduct rheological tests (Sanap and Mohanta 2013). The viscosity of all gels was measured at various rpms, and a relationship was then developed to identify the relationship between shear rate and viscosity. The purpose was to see how the gel behaved at different rates.

2.7.5. Spreadibility

This study is done to examine the extent to which a drug can easily spread when applied to skin using an optimal gel with good spread ability (Khan MFA *et al.,* 2022) (Jain *et al.,* 2010). This experiment is carried out by placing a pre-measured amount of gel on a 1cm circle on glass slides (Atipairin *et al.,* 2020). Now the other slides were pressed on top of it, and a weight of around 0.5 kg was placed on top of it for 5 minutes, and the percent spread of gel was calculated.

2.7.6. Extrudability studies

Extrudability studies were used to determine whether a hydrogel is easily extrudable or not (Abdellatif *et al.,* 2022) (Kaur and Ajitha 2019a). For this, an aluminum collapsible tube was used in which the hydrogel was loaded, and then it was left for ten minutes and checked if the hydrogel could easily extrude from it (Bhardwaj and Singh 2021). The goal was to see if a certain amount of hydrogel could travel through a given cm in a certain amount of time or not.

2.7.7. *Ex vivo* permeation study

Permeation studies were performed by approval of Quaid-I-Azam University, Islamabad bio- ethical committee with reference to No #**BEC-FBS-QAU2022-442**.

The animal (albino mice) was euthanized, and skin was cleaned, hair was removed from the abdominal region of mice using a razor. Hair removal cream was used to strip the remaining tiny hairs (Garg *et al.,* 2016). Then the skin was removed from the abdominal region followed by the removal of subcutaneous tissues with the help of forceps and isopropyl alcohol, to clean fats that were stick to the dermis. The skin was prepared for studies by washing it with PBS 7.4 and then divided it into equal parts. Franz diffusion cell with receptor chamber volume of 5mL and diffusion (0.77 cm^2) was used for studies. The skin was placed in between the receptor and donor chamber facing the subcutaneous side of skin towards donor compartment. After this, the receptor compartment was filled with PBS 7.4. Magnetic stirrer was used to constantly stir the PBS in receptor chamber for the provision of optimal temperature (37±0.5℃). For comparison investigation, simvastatin transfersomes loaded hydrogel and free simvastatin loaded hydrogel were applied to different skin types. Samples were taken from the receptor chamber after 0.5, 1, 1.5, 2, 3, 4, 6, and 12 hours. By using UV-spectroscopy at 280 nm, the drug concentration was determined. After each sample withdrawal, an equivalent volume of new buffer was introduced to the receptor medium in order to maintain sink (Dudhipala *et al.,* 2020, Thakre *et al.,* 2021). To find the results and calculate various permeation parameters like flux (J (µg/cm^2/h)), drug permeated per unit area (µg/cm^2) enhancement ratio (ER) and permeability coefficient (Kp (cm^2/h)) using the following equations:

$$\boldsymbol{J} = \frac{amount\ of\ drug\ \ permeated\ \times Diffusion\ area\ of\ skin}{Time}$$

$$Kp = \frac{1}{Drugi}$$

$$ER = 1/Cr_{form}$$

2.7.8. *In Vitro* release study

Dialysis membrane (12kDa MW, Sigma Aldrich, St. Louis MO, USA) technique were used to perform the drug release study for transfersomes suspension, free simvastatin (control) suspension, simvastatin loaded transfersomal suspension, free simvastatin loaded hydrogel, and simvastatin transfersomal loaded hydrogel were used for the drug release study (Ullah F *et al.,* 2022). Free simvastatin loaded hydrogel was prepared by dispersing free simvastatin (~0.5g) to hydrogel while simvastatin loaded transfersomal hydrogel was prepared by dispersing simvastatin transfersomal

pallets (~0.5 g) in hydrogel. The amount of drug (simvastatin) used in control samples was determined by comparing the encapsulation efficiency of transfersomes. Both ends of the dialysis bag were secured with dialysis closures. Dialysis bags were put in beakers filled with release medium (phosphate buffer) pH 7.4, and 5.5 (Kersani *et al.,* 2020). After that, the beakers were placed in a shaking water bath with a temperature of 37 °C ± 1°C at a speed of 100 rpm. The experiment lasted for 72 hours (Anjani *et al.,* 2021). To prepare the samples, eppendorf tubes (2ml) were used. 2 ml samples were taken at 0.25 ,0.5, 1, 2, 4, 6, 12, 24, 48, 72 hours. To preserve sink conditions, fresh media was replaced in the beaker after each sample withdrawal. UV spectroscopy at 280 nm was used to determine the concentrations of simvastatin in the samples obtained. The drug release was estimated using the results of three trials.

2.7.9. Drug release kinetics

To establish the mechanism and rate of drug release, *in vitro* release data were fitted into a variety of kinetic models, including first order, zero order, korsmeyer peppa's, and the higuchi kinetic model (Mahmood *et al.,* 2023) (Singh and Singh 2020). It was decided to use the top model with the highest R^2 rating (correlation coefficient). The slopes of equations' straight lines were used to calculate the n-values (Diffusion exponent).

2.7.9.1. Zero order model

The definition of a zero-order model is one in which the drug is continuously delivered throughout the drug release mechanism. For this model's release kinetic analysis, the following equation is used:

$$Q_t = Q_0 + K_0 t$$

Where:
Q_t = the amount of medicine released during the course of time Q_0 = Drug concentration at time interval zero
t = time
K_0 = zero order constant

2.7.9.2. First order model

A model of drug release where the rate is directly proportional to the drug concentration is known as first order kinetics. We used this equation to include the release kinetics to show that our investigation follows first order kinetics.

$$Log\ C = log\ C0 - K1t/2.303$$

Where:
K =first order kinetics
C_0 = quantity of drug at zero interval C =remaining drug

2.7.9.3. Higuchi model

The Fick's law of diffusion, which governs how drugs diffuse from their matrix systems, is followed by this model. In order to ascertain that, this study used the Higuchi model, and the release kinetics data was entered into the equation.

$$\boldsymbol{ft = Q = A\sqrt{D\,(2C - C_s)\,C_{st}}}$$

$$CSt\ ft = Q = KH\ x\ t1/2$$

where:
K_H = Higuchi constant
Cs = drug matrix solubility
C= amount of drug at zero-time interval Q= quantity of drug release
D =amount of drug diffuses

2.7.9.4. Korsmeyer-peppas model

This model is also called as extension of *Higuchi model*. When it's confirmed that the release of drug follows diffusional pattern, so this model applies to confirm which type of diffusion was followed. Following equation are used to analyze it.

$Mt/M \infty = K_{kp} x t^n$

where
K_{kp} = release constant
n = diffusional exponent

"n" describes the mechanism through which drug release i.e., if value of n less than or equal to 0.45, which indicate the Fick's law diffusion, and the range between 0.45<n>0.89 indicates non-Fick's law diffusion.

2.8. Skin Irritation Studies

Skin irritation test was used to check if the formulation is safe to use on the skin (Gupta *et al.,* 2022). This was done by conducting *Draize Patch Test*. The albino mice's skin was shaved on the dorsal side 24 hours prior to study (Khan MA *et al.,* 2015) (HU *et al.,* 2021). 4 animal groups were made. Group 1 (control group) was not given any treatment. Group 2 (negative control) was given 0.8 % solution of formalin. Group 3 was treated with free simvastatin loaded hydrogel. Group 4 was treated with simvastatin loaded transfersomal hydrogel (Asad et al. 2021). Free simvastatin loaded hydrogel was prepared by dispersing free simvastatin (~0.5g) to hydrogel while simvastatin loaded transfersomal hydrogel was prepared by dispersing simvastatin transfersomal pallets (~0.5 g) in hydrogel. After 6, 12, and 24 hours, the

erythema and edema score were calculated using the standard Draize scoring system and the primary cutaneous irritant index.

2.9. *In Vivo* Studies

2.9.1. Animal selection

Male sprague dawley rats were used to evaluate antihyperlipidemic activity and antiatherosclerosis activity of simvastatin transfersomes loaded transdermal hydrogel for transdermal delivery (Rizvi *et al.,* 2019). Animals (weigh 250 ±50 g) were purchased from Riphah International University (Islamabad, Pakistan). Animals were kept under controlled environment comprising 40% - 60 % relative humidity, with alternate dark-light cycles, 25±1℃ temperature, with high fatty cholesterol diet (Kaur and Ajitha 2019b) (Ullah *et al.,* 2016). The Quaid-i-Azam University Bioethical Committee approved all animal studies in accordance with NIH rules and the Animal Welfare Act.

2.9.2. Hyperlipidemia induction and treatment with simvastatin transfersomal hydrogel

A significant risk factor for atherosclerosis is hyperlipidemia. Simvastatin transfersomes based transdermal hydrogel's anti-atherosclerotic effect was investigated using the poloxamer 407 induced hyperlipidemia model of rat (Belguith-Hadriche *et al.,* 2016) (Palmer *et al.,* 1997). 16 rats were chosen and distributed at random. A total of 4 groups were formed based on treatment procedure. Each group contain 4 rats. Group 1 (Normal) was given normal saline via intraperitoneal injection. Group 2 (Negative) was given poloxamer 407 intraperitoneal injection as an inducer only. Group 3 consists of rats to which poloxamer 407 intraperitoneal injection (inducer) and free drug (simvastatin) loaded gel (treatment) (El-Say *et al.,* 2020) are given. Group 4 (treatment) was poloxamer 407 intraperitoneal injection (inducer) and simvastatin loaded transfersomes based transdermal hydrogel. Before the start of studies all the animals were kept fasting for 12 hours prior to induction of poloxamer 407 or normal saline. Treatment was given after 12 hours, after injection of inducer or normal saline, (as in case of Group 3 and Group 4). Blood samples were collected via the cardiac puncture after anesthetizing with the combination of ketamine and xylazine (anesthetics) at 16±60 mg respectively, samples were withdrawn at 0, 12, 24, 36, 48 and 72 hours after the

intraperitoneal injection of saline and poloxamer 407. To obtain the serum, blood was taken, maintained at room temperature for 10 to 15 minutes, and then centrifuged at 3,000 rpm for 10 minutes at 4 °C. Then, for additional analysis, these serum samples were collected and stored in the refrigerator at -20 ºC for further analysis.

2.9.3. Biochemical serum analysis

After the collection of serum samples, these were analyzed spectrophotometrically. For estimation of high-density lipoprotein cholesterol (HDL-c), homogeneous enzymatic calorimetric assay with reagent kit was used. And for total cholesterol estimation, enzymatic calorimetric assay and suitable reagent kit was used. Low density lipoprotein (LDL) and very low density lipoproteins (vLDL) were calculated by Friedwald formulas (Singh *et al.,* 2020). i.e.

$$vLDL = \frac{TG}{5}$$

$$LDL = Total\ Cholesterol - (vLDL + HDL)$$

Atherogenic index (Abid *et al.,* 2021) were calculated using the following equation

$$Atherogenic\ Index = \log(\frac{TG}{HDLC})$$

2.10. Stability Studies

Stability tests were carried out by placing the optimized formulation to three temperature ranges: 4ºC±2 ºC, 25 ºC ±2 ºC and 40 ºC ±2 ºC for nearly 6 months (Parhi and Padilam, 2018). Following tests were performed to determine that our formulation was stable over time, such as physical appearance (like phase separation, color change and grittiness), pH, drug content (%), Particle size (nm), and entrapment efficiency (%) over different time periods.

2.11. Statistical Analysis

Statistical Analysis was performed using the ANOVA with subsequent multiple comparison test. Mean and Standard deviation was used to present data. Significance level was set at $p<0.05$.

CHAPTER 3

RESULTS

3. RESULTS

3.1. Preparation of Simvastatin Loaded Transfersomes

Simvastatin loaded formulations were successfully prepared by using thin film hydration procedure with a little modification.

3.2. Optimization of Transfersomal Formulation

Design Expert Software was used for the optimization of formulation. Using the Box Benchen Model, it gave 14 formulations, their responses were inserted in this software.

Table3.1. Optimization table for simvastatin transfersomal formulation.

Runs	PL (mg)	S (mg)	Drug (mg)	PS (nm)	ZP (mV)	PDI	EE (%)
1	82.5	25	15	114	-23.1±0.84	0.23±1.96	86.23±0.75
2	82.5	10	15	136	-11.2±0.5	0.309±0.40	74.3±0.64
3	90	25	12.5	125	-24.4±1.44	0.198±1.92	88.87±1.25
4	75	25	12.5	109	-19.8±1.54	0.238±0.68	89.6±1.23
5	82.5	25	10	111	-22.6±2.25	0.295±0.26	89.53±1.84
6	90	10	12.5	147	-11.4±0.77	0.165±0.69	68.2±0.67
7	82.5	17.5	12.5	117	-14.2±1.69	0.176±0.48	86.5±0.55
8	90	17.5	10	138	-22.1±2.41	0.18±0.82	76.03±0.89
9	90	17.5	15	139	-22.5±1.95	0.175±0.69	76.58±0.44
10	82.5	10	10	134	-10.4±0.91	0.28±0.58	85.96±1.8
11	75	17.5	10	113	-16.2±1.52	0.258±0.94	89.6±0.45
12	82.5	17.5	10	117	-18.8±2.64	0.244±0.43	84.31±1.4
13	75	17.5	15	116	-17.6±1.56	0.255±0.91	90.44±1.64
14	75	10	12.5	123	-10.2±1.94	0.246±0.73	89.27±0.36

3.3. Effects of Surfactant, Phospholipid and Drug on Different Parameters

3.3.1. Effect of surfactant, phospholipid, and drug on particle size

The 3D graphs demonstrate the impact of various variables on particle size as shown in Figure 3.1. Particle size decreases with increasing surfactant concentration and vice versa, which may be because a higher surfactant concentration encourages micelle formation rather than vesicle formation. Particle size increases with an increase in phospholipid content. While drug concentration increased, there was no discernible change in particle size.

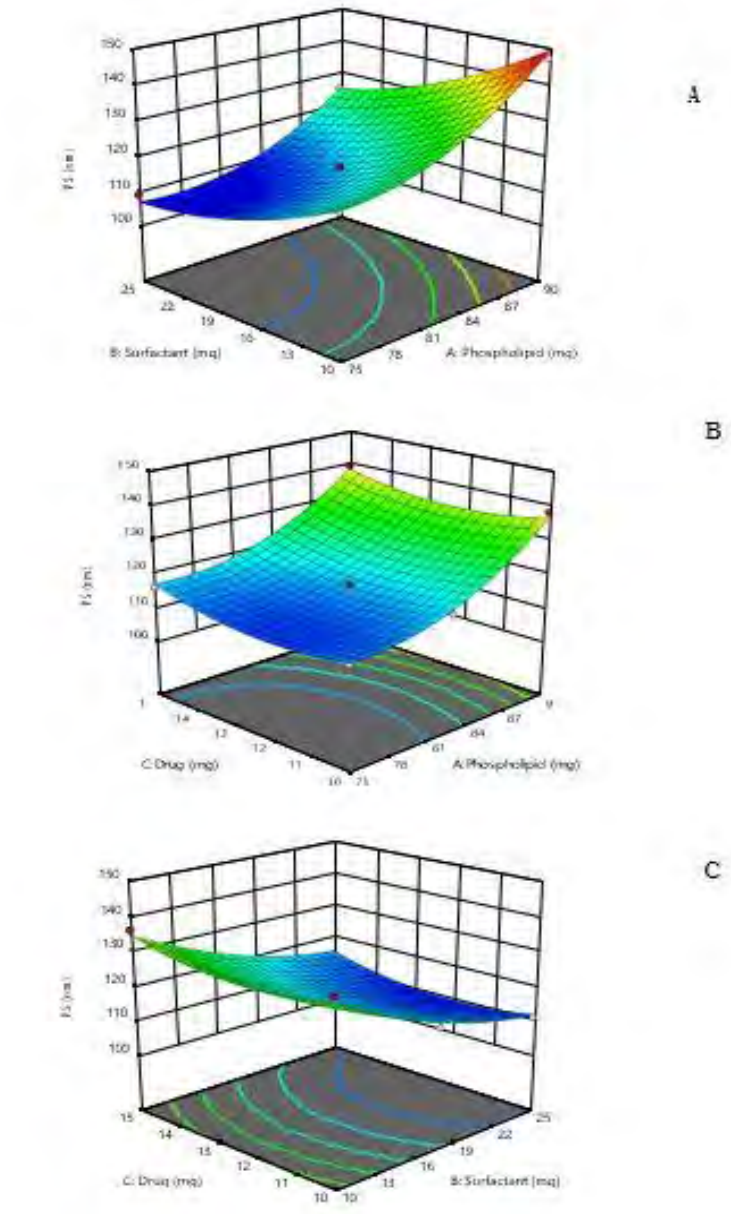

Figure 3.1. Effect of surfactant, phospholipid, and drug on particle size.

3.3.2. Effect of surfactant, phospholipid, and drug on zeta potential

The effect of surfactant and phospholipid on zeta potential is depicted in the 3D graph of Figure 3.2. Raising the phospholipid concentration causes the zeta potential to decrease and vice versa. Increase in surfactant concentration causes increase in zeta potential which results in decrease in vesicle aggregation and increase in stability. Whereas increasing the simvastatin concentration causes decrease in zeta potential.

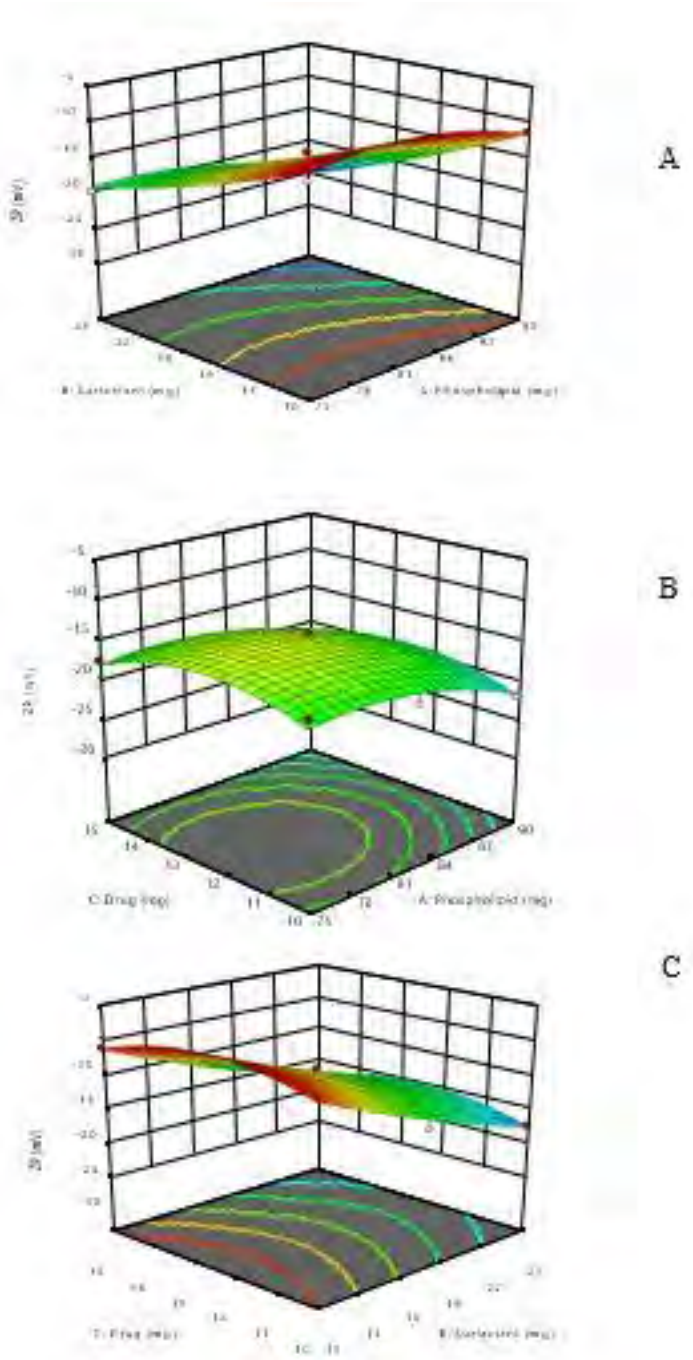

Figure 3.2. Effect of surfactant, phospholipid, and drug on zeta potential.

3.3.3. Effect of surfactant, phospholipid, and drug on entrapment efficiency

According to the 3D graphs of Figure 3.3, entrapment effectiveness decreases as surfactant concentration increases. Additionally, a rise in phospholipid concentration results in a decline in entrapment effectiveness. Entrapment efficiency rises with drug concentration and then decreases.

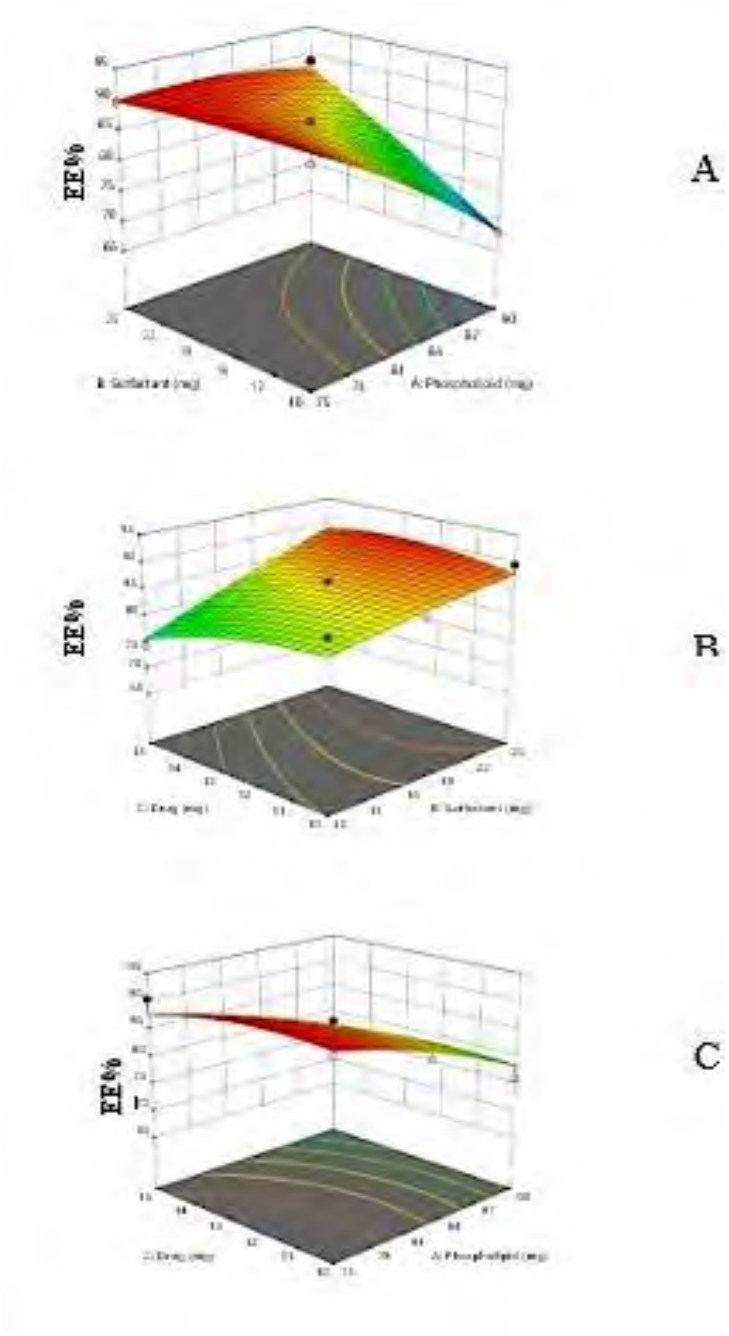

Figure 3.3. Effect of surfactant, phospholipid, and drug on entrapment efficiency.

3.3.4. Effect of lipid and surfactant on poly dispersity index

The graphs of Figure 3.4, obtained through the design expert shows by increasing the surfactant concentration, poly dispersity index decreases, and by increasing the concentration of surfactant, polydispersity index decreases and vice versa. Increasing the drug concentration, poly dispersity index increases.

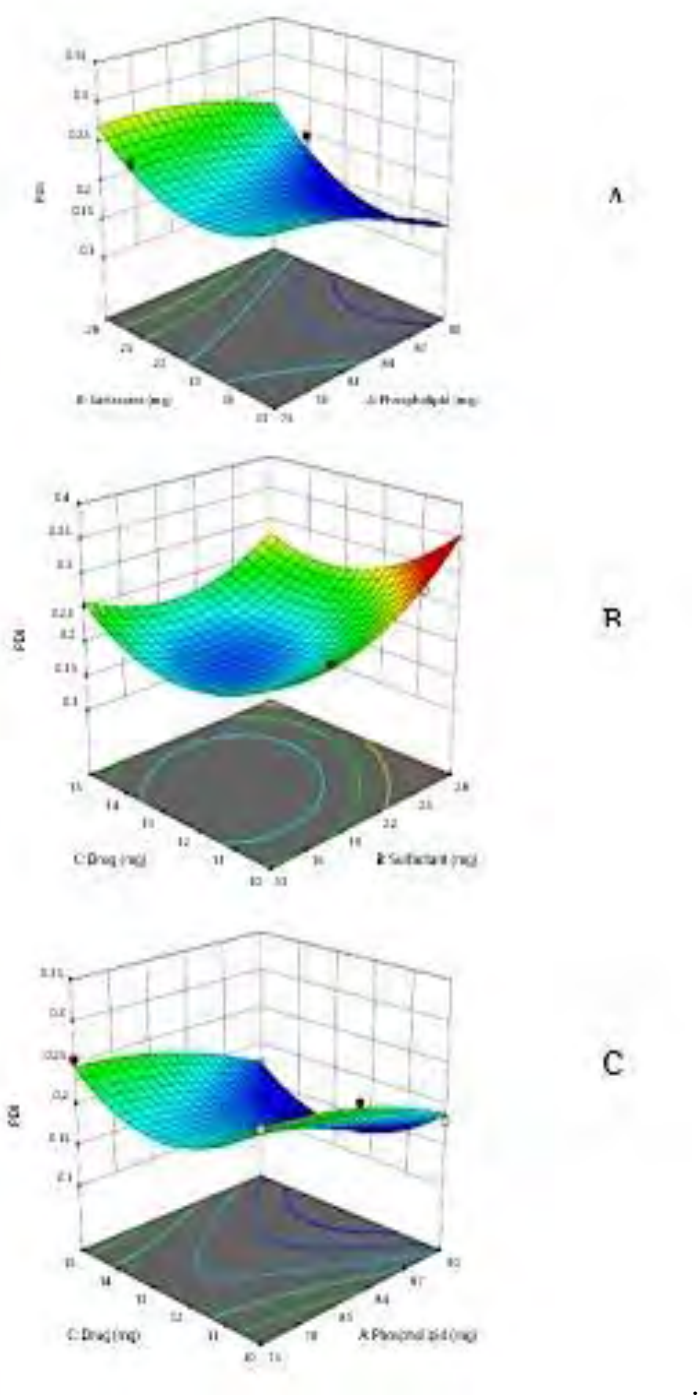

Figure 3.4. Effect of surfactant, phospholipid, and drug on PDI.

3.4. Characterization of Transferosomes

3.4.1. Particle size and PDI

Overall vesicle size, Poly Dispersity Index, and Zeta Potential of the optimized formation were assessed using Zeta Sizer. Figure 3.5 illustrates the improved formulation, which had a PDI of 0.273, a vesicle size of 128.7 nm, and a Zeta potential of -20.4 mV.

3.4.2. Particle size and particle charge

Dimensions and charge of the particles Vesicles are more stable due to surface charge. According to the Figure 3.5, the improved formulation's zeta potential was -20.4 mV. Particle size of the optimized formulation was 128.7 nm as opposed to 126 nm for the blank.

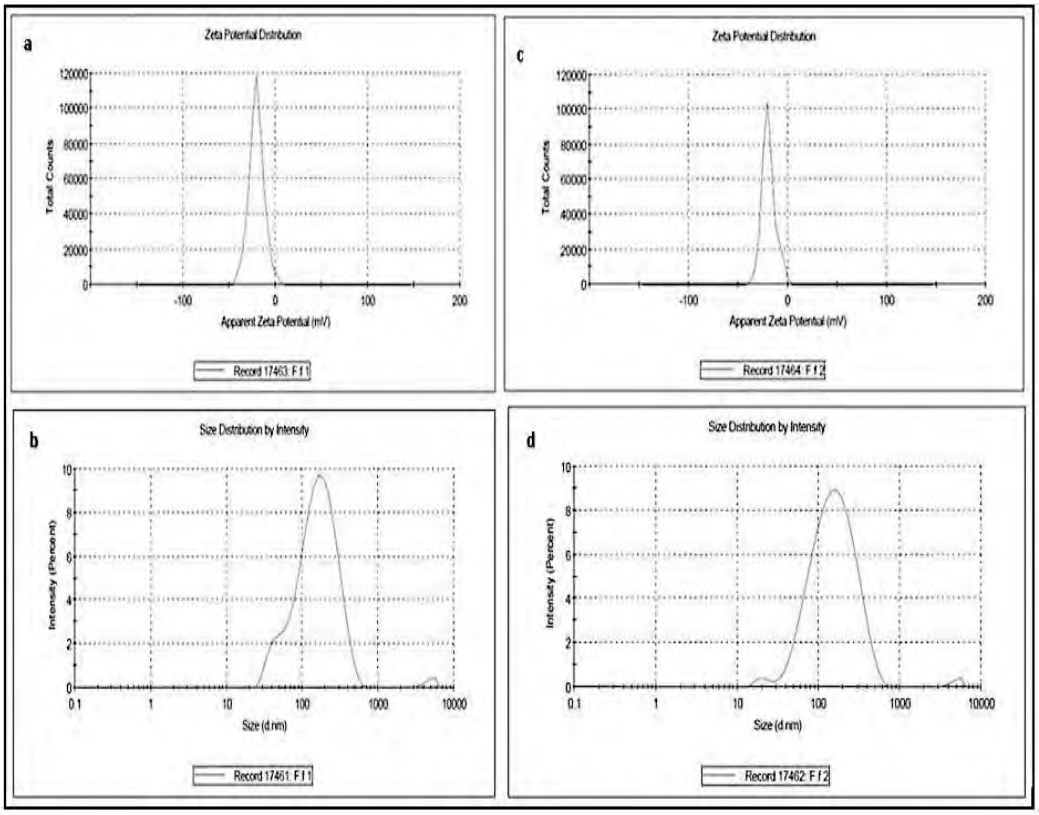

Figure 3.5. Characterization of optimized formulation.
Note: *(a) Zeta potential of blank formulation, (b) size distribution of blank formulation (c) zeta potential of transfersomal formulation, (d) size distribution of transfersomal formulation.*

3.4.3. Simvastatin calibration curve

Simvastatin showed linear regression curve at the following absorbance as shown in table 3.2.

Table 3.2. Absorbance value of different concentration of simvastatin in distilled water.

S no.	Concentration (µg/ml)	Absorbance
1	0.5	1.152
2	1	1.233
3	2	1.351
4	4	1.589
5	6	1.766
6	8	2.01
7	10	2.25
8	12	2.582

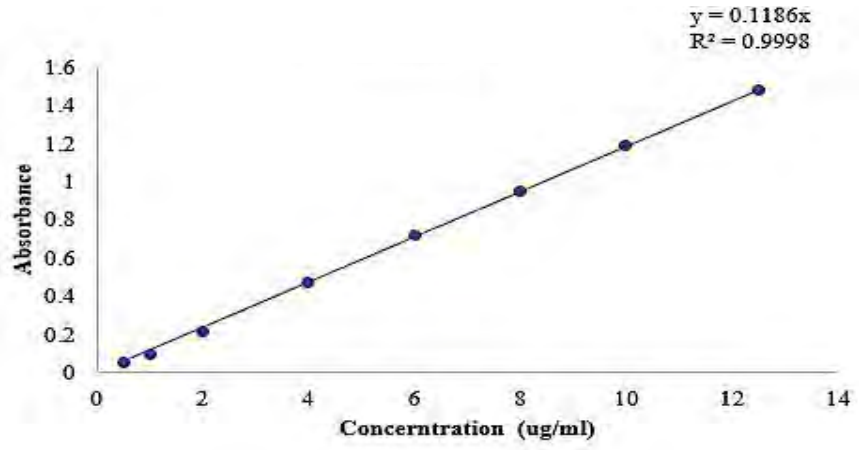

Figure 3.6. Standard calibration curve of simvastatin.

3.4.4. FTIR analysis

The optimized formulation's spectrogram as shown in Figure 3.7 and table 3.3. revealed that the peaks were unaltered, showing that there was no drug-transfersome interaction. The free simvastatin showed various characteristics peaks i.e., 3454 cm^{-1} for hydroxyl group, 3010 cm^{-1} for alkene, 2810 cm^{-1}, and 1700 cm^{-1} for lactone, 1164 cm^{-1} and 1066 cm^{-1} for carbonyl groups. Similarly, in case of phospholipid peaks of alkene were shown at 2918 cm^{-1} and 2850 cm^{-1}, carbonyl at 1738 cm^{-1}, P=O at 1236 cm^{-1} and $N(CH_3)_3$ at 970 cm^{-1}. tween 80 showed peaks at 3454 cm^{-1} for hydroxyl, 1738 cm^{-1} for carbonyl, 972 cm^{-1} for P=O and 1087 cm^{-1} for C-O-C. These peaks were present in the final FTIR formulation, demonstrating that there was no appreciable interaction between the free simvastatin and formulation excipients.

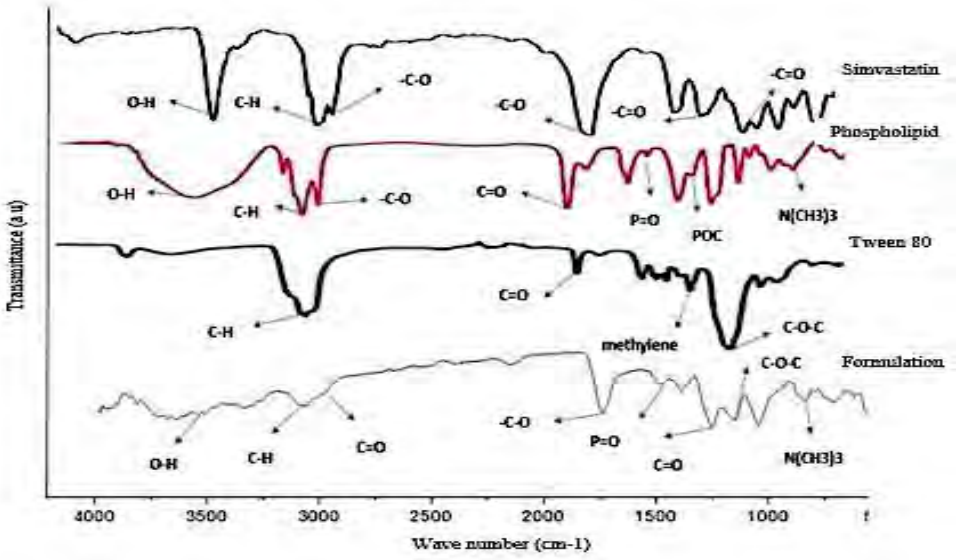

Figure 3.7. FTIR analysis.

Table 3.3. FTIR spectrum peak analysis.

Functional Groups	Simvastatin	Phospholipid	Tween 80	Formulation
OH stretch	3454		3454	3454
C-H stretch	3010	2918, 2850		3010
C-O stretch	2810 1700	1738	1738	2895 1656
C=O stretch	1264			1268
P=O	------	1236	972	1230
N(CH₃) ³	-------	970		970
C-O-C	-------		1087	1076

3.4.5. XRD analysis

The amorphous nature of optimized lyophilized formulation was further confirmed by the XRD diffractogram. Simvastatin showed number of peaks at 10.88, 15.57, 17.17 ,19.32 indicating crystalline nature of simvastatin, whereas in the optimized lyophilized formulation these peaks have diminished due to amorphous nature of formulation, as shown in Figure 3.8.

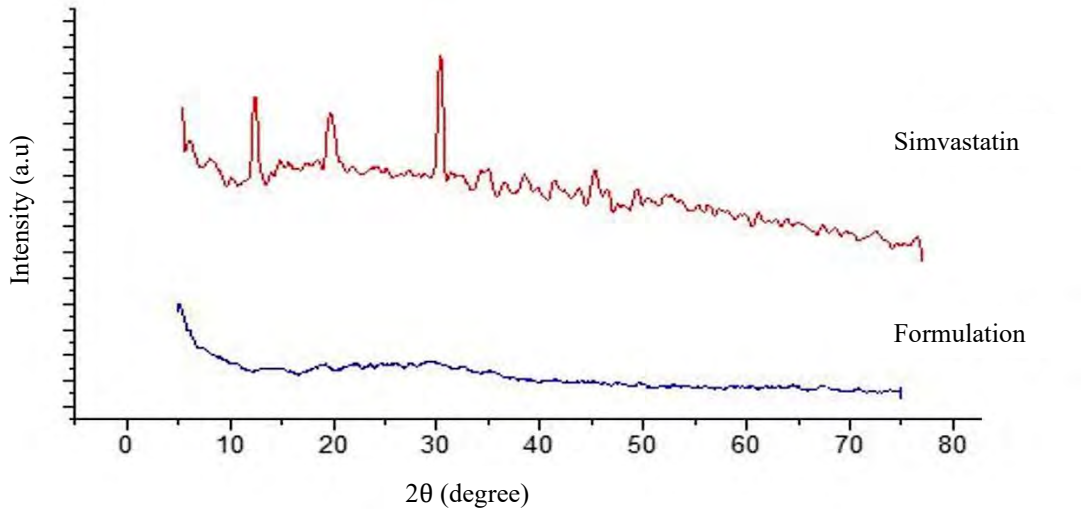

Figure: 3.8. XRD analysis of simvastatin loaded transfersomes.

3.4.6. DSC analysis

DSC thermogram simvastatin showed sharp endothermic peak at 140℃ (Abo-zalam *et al.* 2021) indicating crystallinity of simvastatin. Similarly, phospholipid and tween 80 showed sharp peak at 232.9℃ and 113.8℃ respectively. These sharp endothermic peaks disappeared in the optimized formulation, as shown in graph of Figure 3.9, indicating the conversion of crystalline nature of simvastatin to amorphous form after the transfersomes formation.

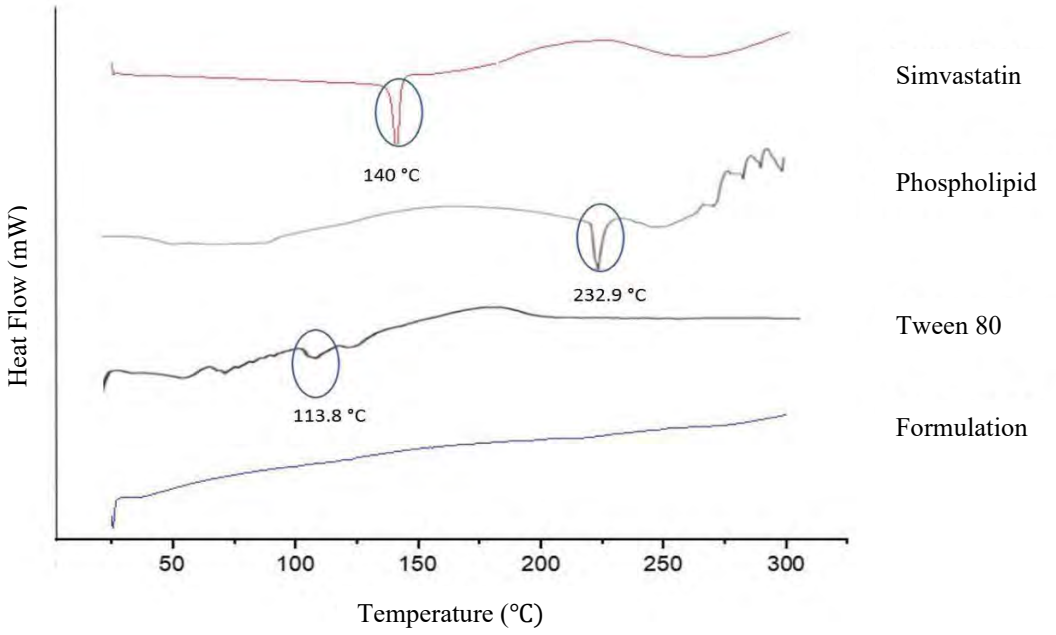

Figure: 3.9. DSC analysis of simvastatin loaded transfersomes.

3.5. Characterization of Carbopol Hydrogel

Table 3.4 shows the different characterizations for gel ratios. The findings show that using Carbopol 1% ratio produces the best results. White in color and homogenous, pH 5.6, which is acceptable for skin application, high drug content uniformity of about 83.1 %, Gel exhibits non-Newtonian behavior (shear thinning), and has a high spread ability of about 387%, and the gel is easily extrudable from container.

Table 3.4. Characterization of simvastatin loaded transfersomal hydrogel.

Factors	**Hydrogel 1%**
Physical Appearance	White color, homogenous
pH value	5.6
Drug Content	83.1%
Behavior	Non-Newtonian shear thinning
Spreadibility	387%
Extrudability	Excellent

The table 3.4 shows the different characterizations for gel ratios. The findings show that using Carbopol 1% ratio produces the best results. White in color and homogenous, pH 5.6, which is acceptable for skin application, high drug content uniformity of about 83.1 %, hydrogel exhibits non-Newtonian behavior (shear thinning), and has a high spread ability of about 387%, and the gel is easily extrudable from container.

3.5.1. Rheological study of hydrogel

Rheological studies were performed to analyses the behavior of simvastatin loaded Transfersomal based transdermal hydrogel at different shear rates. The graph of Figure 3.10 illustrates that these hydrogel does not follow Newtonian law as its viscosity decreases with the increase in the shear rate which is one of the prominent characteristics of the smooth hydrogel.

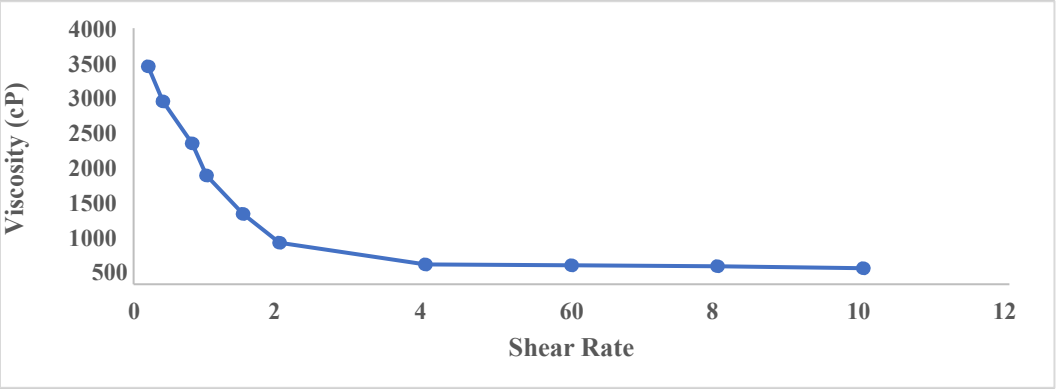

Figure 3.10. Carbopol based hydrogel viscosity versus shear rate.

3.6. Optimized Formulation Released Study

3.6.1. *In vitro* release study of simvastatin at pH 7.4 and 5.5

In vitro drug release was performed to observe the release of simvastatin from transfersomal hydrogel under standardized conditions. As shown in Figure 3.11 and Figure 3.12, free simvastatin, almost 80 % of simvastatin was released within first 12 hours in both medias. Burst release of simvastatin was observed in 7.4 pH medium. Within 2 hours almost 60 % of simvastatin was released and within 36 hours 80 % of simvastatin was released from transfersomal suspension. However, simvastatin loaded transfersomal hydrogel showed the burst release of 28 % within 2 hours and almost 60 % of simvastatin was released in 36 hours in sustained manner and almost 70 % in 72 hours. At pH 5.5 a very small amount of simvastatin was released from transfersomal suspension and hydrogel. As all results which studied on pH 7.4 show, transfersomal hydrogel is quite stable and produce sustained effects for long period of time.

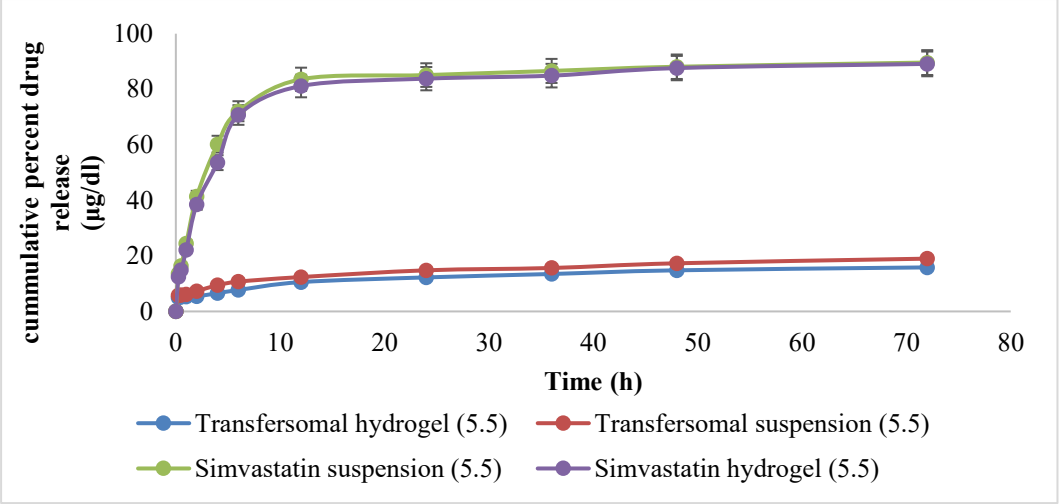

Figure: 3.11. *In vitro* release study of simvastatin loaded transfersomes at pH 5.5.

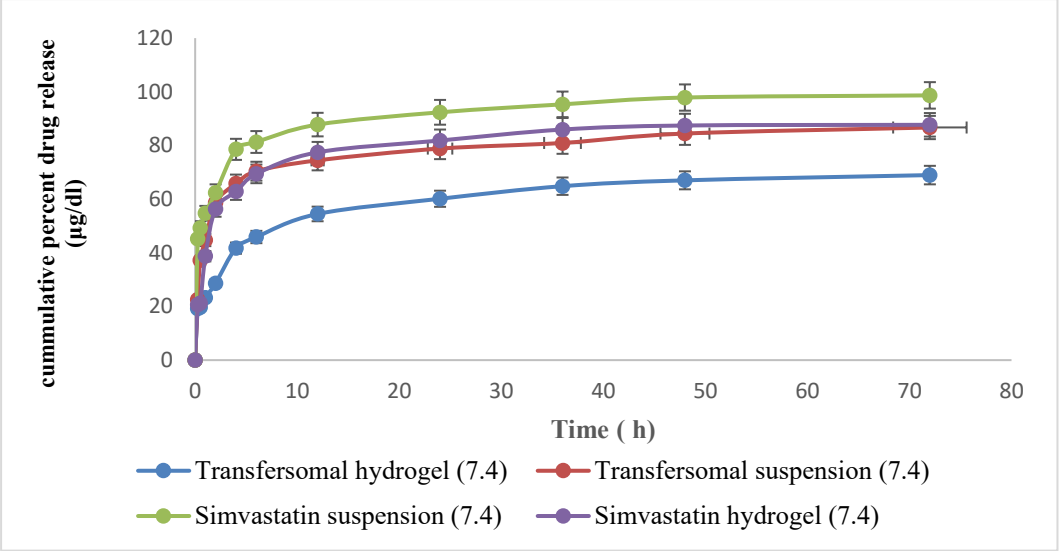

Figure 3 . 1 2 . *In vitro* release study of simvastatin loaded transfersomes at pH 7.4.

3.6.2. Kinetics models

The best fit model based on a high R^2 value was Korsmeyer peppas, which was utilized to estimate the mechanism of drug release from the optimized transfersomal hydrogel as shown in table 3.5.

Table 3.5. Kinetic models of drug release.

Model	**Zero Order**		**First Order**		**Higuchi**		**Korsmeyer Peppas**	
	k	r^2	k	r^2	K	R^2	n	r^2
Simvastatin loaded Transfersomal Formulation	1.874	0.9090	0.419	0.9322	15.603	0.9216	0.157	0.9971
Simvastatin loaded Transfersomal Hydrogel	1.848	0.9261	0.548	0.9184	15.266	0.9585	0.163	0.9993

3.7. *Ex Vivo* Permeation Studies

Simvastatin-loaded transfersomal hydrogel and free simvastatin hydrogel were the subjects of diffusion investigations, which revealed that generated transfersomes had much higher transdermal permeation (745 µg/cm^2) than free simvastatin hydrogel (127 µg/cm^2), as shown in Figure 3.13.

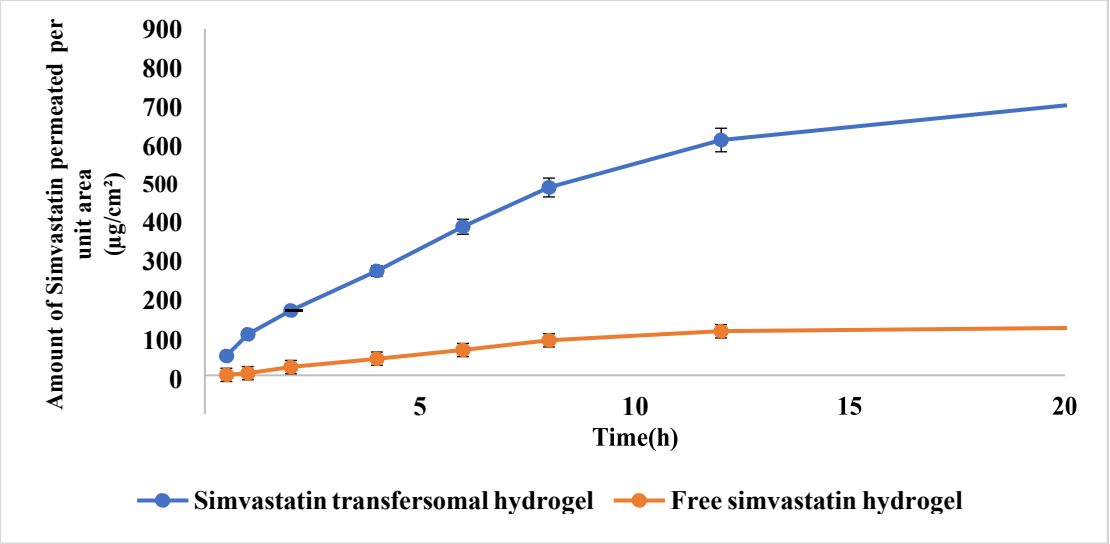

Figure 3.13. *Ex vivo* permeation study of simvastatin loaded transfersomes loaded hydrogel versus simvastatin raw hydrogel.

3.8. Histopathology Skin Irritation Study of Optimized Hydrogel

Histopathological studies showed that there was no damage to the surface of skin tissues or skin layers in mice after administration of free simvastatin treated hydrogel group and simvastatin loaded transfersomal treated hydrogel group as shown in Figure 3.14. No diffusion of neutrophils or infiltration of T-cells was observed. These results indicate that simvastatin loaded transfersomal treated hydrogel is safe for transdermal administration.

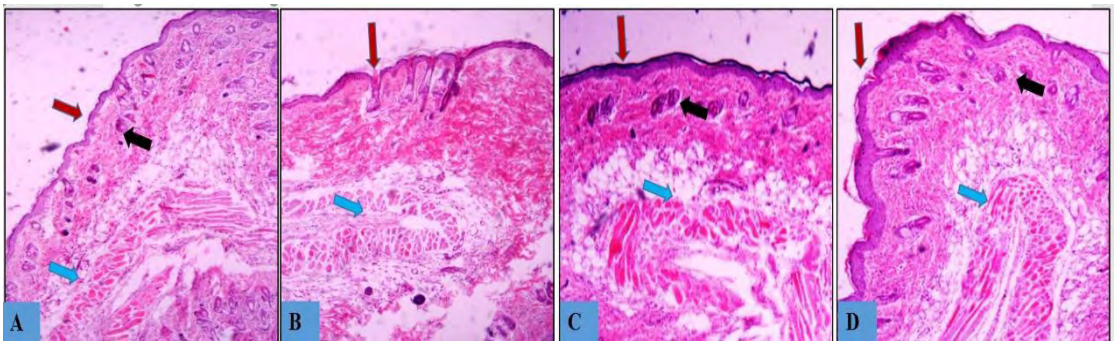

Figure 3.14. Histopathological studies on mice.
Note: *shows the erythema and edema score on different groups shows the reaction of normal mice (A), formalin treated mice (B), free simvastatin loaded hydrogel treated mice (C), simvastatin loaded transfersomal hydrogel treated mice (D).*

3.9. Draize Scoring Evaluation of Skin Irritation Study

Skin irritation study of optimized formulation based on Draize scoring evaluation and by dermal irritation index showed no major skin reaction after the administration of simvastatin loaded transfersomal hydrogel. From Figure 3.15, b, it is noted that in formalin treated group, erythema and edema was observed with change in skin integrity, while in case of free simvastatin treated hydrogel group and simvastatin loaded transfersomal hydrogel treated group there was no obvious change in skin as compared with the normal group.

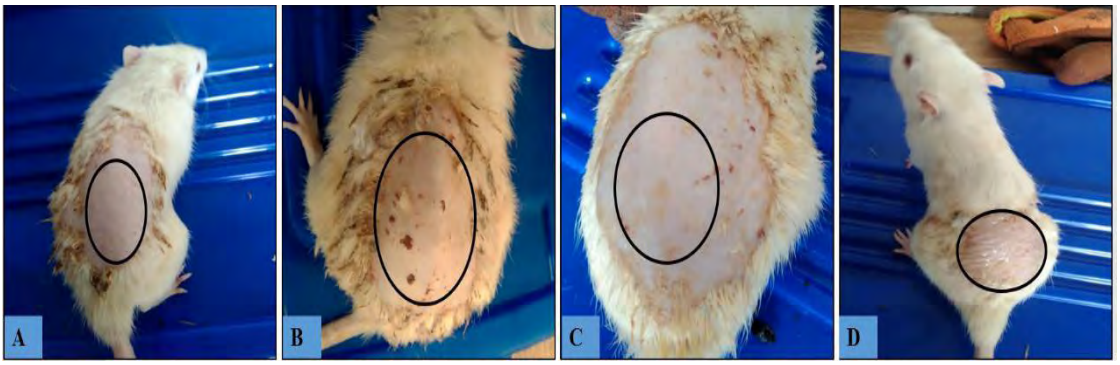

Figure 3.15. Skin irritation studies on mice.
Note: *(A) shows the reaction of normal mice, (B) formalin treated mice, (C) free simvastatin loaded hydrogel treated mice, (D) simvastatin loaded transfersomal hydrogel treated mice.*

Table 3.6. Erythema and edema score on different groups of mice.

Evaluation and Assessment of Skin Erythema Score	**06 hr**	**12 hr.**	**24 hr.**	**Evaluation and Assessment of Skin Edema Score**	**06 hr.**	**12 hr.**	**24 hr.**
Normal Control	0	0	0	Normal Control	0	0	0
Negative Control	1	1	2	Negative Control	1	1	1
Free simvastatin loaded hydrogel treated Group	0	0	0	Free simvastatin loaded hydrogel treated Group	0	0	0
Simvastatin loaded transfersomal hydrogel treated Group	0	0	0	Simvastatin loaded transfersomal hydrogel treated	0	0	0

3.10. Stability Studies

Stability studies were performed to check if the hydrogel was stable at different temperature ranges or not as shown in table 3.7. Hydrogel was placed in different temperatures i.e., 4°C ± 2°C, 25°C ± 2°C, and 40°C ± 2°C for 6 months. Different parameters like physical appearance, pH, drug content, particle size and entrapment efficiency were analyzed, and results were compiled in form of

table as shown in table 3.8. Results showed that the transdermal hydrogel remain stable for 6 months at different temperatures.

Table 3.7. shows stability profile of simvastatin loaded transfersomal hydrogel.

Time (months)	Temperature (°C) ±2°C	Physical Appearance			pH ±1	Drug Content (%)	Particle Size (nm)	EE (%)
		Phase separation	Color Chang	Grittiness				
0	4	No	No	Smooth	5.6	93.1	128	89
	25	No	No	Smooth	5.6	93.1	128	89
	40	No	No	Smooth	5.6	93.1	128	89
1	4	No	No	Smooth	5.7	92.58	128.5	88.85
	25	No	No	Smooth	5.7	92.58	128.5	88.85
	40	No	No	Smooth	5.7	92.56	128.5	88.85
3	4	No	No	Smooth	5.8	92.3	129.5	88
	25	No	No	Smooth	5.8	92.3	129.5	88
	40	No	No	Smooth	5.8	92.3	129.5	88
6	4	No	No	Smooth	5.8	91.84	130	87.5
	25	No	No	Smooth	5.8	91.84	130	87.5
	40	No	No	Smooth	5.8	91.82	130	87.5

3.11. *In Vivo* Studies

Hyperlipidemia is a major risk factor of atherosclerosis, Poloxamer 407 induced hyperlipidemic rat model was used to analyze the antiatherosclerosis potential of simvastatin transfersomes. Because TG, TC, HDL-c, and LDL levels rise in hyperlipidemia, serum samples were taken and tested for these markers as well as the atherogenic index and the following findings were made as shown in table 3.8.

Table 3.8. Effect of simvastatin loaded transfersomal hydrogel on Serum lipid and lipoprotein levels in P-407 induced hyperlipidemic rat model and Atherogenic Index.

Parameters	Time (h)	Normal Control	Disease Control	Free simvastatin loaded hydrogel treated	Simvastatin loaded transfersomal hydrogel treated
HDL-C (mg/dL)	0	44.84 ± 1.65	45.05 ± 1.10	45.30 ± 1.05	45.26 ± 1.30
	24	45.10 ± 1.34 [a]	39.24 ± 1.27 [b]	37.80 ± 1.26	38.50 ± 1.34
	48	45.23 ± 0.67 [a]	30.15 ± 0.40 [b]	38.10 ± 0.55 [a]	40.50 ± 0.34 [a]
	72	45.67 ± 1.07 [a]	27.28 ± 1.15[b]	39.45 ± 1.26 [a]	42.14 ± 1.20 [a]
VLDL-C (mg/dL)	0	22.50 ± 1.40	23.06 ± 0.65	22.75 ± 0.50	23.00 ± 0.15
	24	22.80 ± 0.15[a]	45.25 ± 0.10[b]	44.25 ± 0.24	42.15 ± 0.40 [a]
	48	23.15 ± 0.20 [a]	48.25 ± 0.29[b]	41.50 ± 0.50 [a]	33.24 ± 0.20[a]
	72	24.05 ± 0.50 [a]	52.50 ± 0.20 [b]	38.18 ± 0.35 [a]	31.26 ± 0.27 [a]
TC (mg/dL)	0	82.23 ± 2.40	85.5 ± 3.10	88.25 ± 3.10	86.56 ± 0.78
	24	85.30 ± 1.05 [a]	710.20 ± 3.25 [b]	690.45± 1.05	720.45 ± 2.25[a]
	48	88.50 ± 1.25 [a]	715.45 ± 2.75 [b]	620.50 ± 2.45 [a]	540.70 ± 2.25[a]
	72	90.22 ± 1.10 [a]	750.25 ± 2.50 [b]	600.32 ± 0.59 [a]	400.35 ± 1.42 [a]
TG (mg/dL)	0	75.35 ± 4.65	82.00 ± 2.15	90.20 ± 2.02	84.40 ± 1.10

	24	80.23 ± 2.15 [a]	350.63± 2.45 [b]	360.50 ± 1.90	355.30± 1.70 [a]
	48	76.40 ± 4.50 [a]	440.50 ± 2.28 [b]	310.10 ± 2.29 [a]	245.80 ± 2.40 [a]
	72	78.90 ± 3.15 [a]	560.23 ± 1.80 [b]	280.50 ± 1.45 [a]	220.50 ± 1.10 [a]
HDL/LDL ratio	0	1.99	1.95	1.99	1.96
	24	1.978	0.87	0.94	1.009
	48	1.95	0.62	1.06	1.21
	72	1.89	0.52	1.12	1.267
Atherogenic Index	0	0.2	0.26	0.299	0.269
	24	0.25	0.95	0.979	0.922
	48	0.22	1.16	0.91	0.783
	72	0.23	1.31	0.795	0.518

Note: *'a' shows the comparison of the values with the normal range, 'b' shows the comparison of the values with the normal range.*

The intraperitoneal injection of poloxamer increased the levels in first 24 hours remarkedly in all groups. Poloxamer 407 in diseased control raised the TC level from 85.5 mg/dl to 710 mg/dl in first 24 hours and to 750 mg/dl in 72 hours. The levels of vLDL in serum was increased from 23 mg/dl to 45 mg/dl in 24 hours and to 520 mg/dl in 72 hours.

In HDL-c graphs as shown in Figure 3.16, diseased control decreases significantly from 45 mg/dl to 27 mg/dl in 72 hours. Free simvastatin hydrogel and simvastatin loaded transfersomal hydrogel showed increase in HDL concentration i.e., from 37 mg/dl to 39 mg/dl and 42 mg/dl after 72 hours.

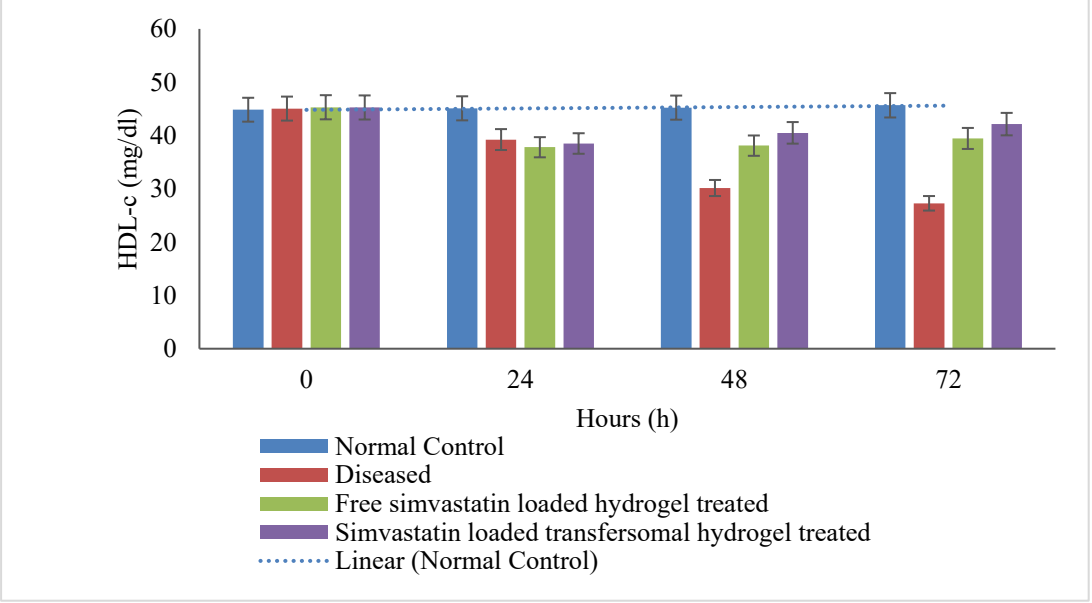

Figure 3.16. HDL-c levels in different groups of rats.

In vLDL graphs as shown in Figure 3.17, diseased control increases significantly from 23 mg/dl to 45 mg/dl in 24 hours and to 520 mg/dl in 72 hours. Free simvastatin hydrogel and simvastatin loaded transfersomal hydrogel showed

decrease in vLDL concentration i.e., from 44 mg/dl to 38 mg/dl and 31 mg/dl after 72 hours.

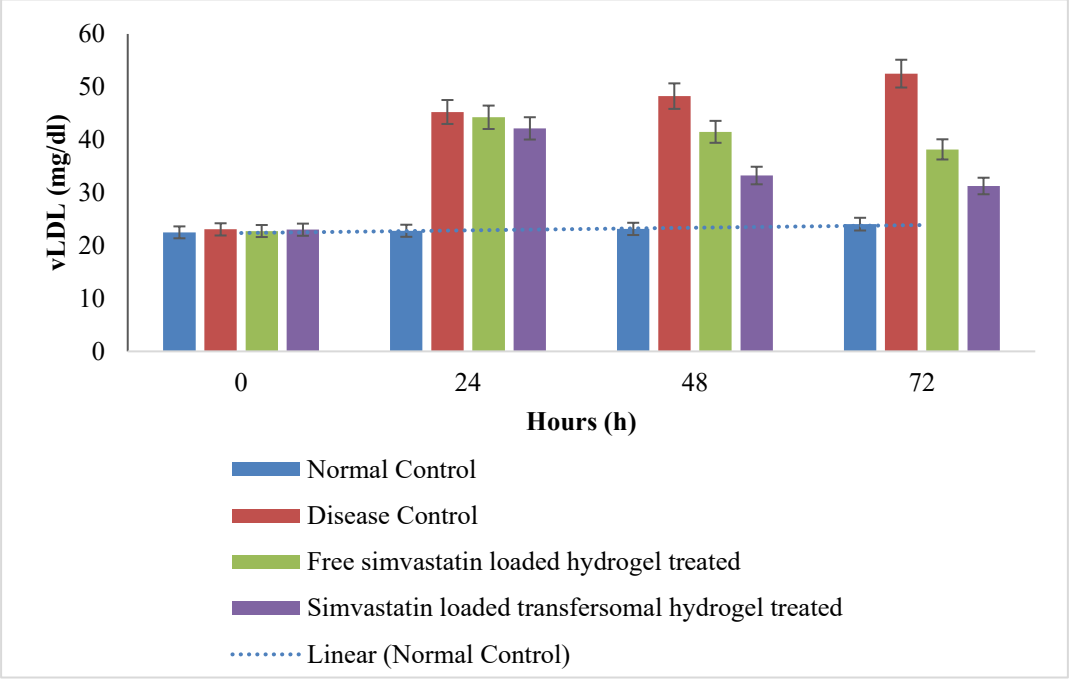

Figure 3.17. vLDL levels in different groups of hyperlipidemic rats.

In TC graphs as shown in Figure 3.18, diseased control increases significantly from 85.5 mg/dl to 710 mg/dl in first 24 hours and to 750 mg/dl in 72 hours. Free simvastatin hydrogel and simvastatin loaded transfersomal hydrogel showed decrease in TC concentration i.e., from 650 mg/dl to 600 mg/dl and 400 mg/dl respectively after 72 hours.

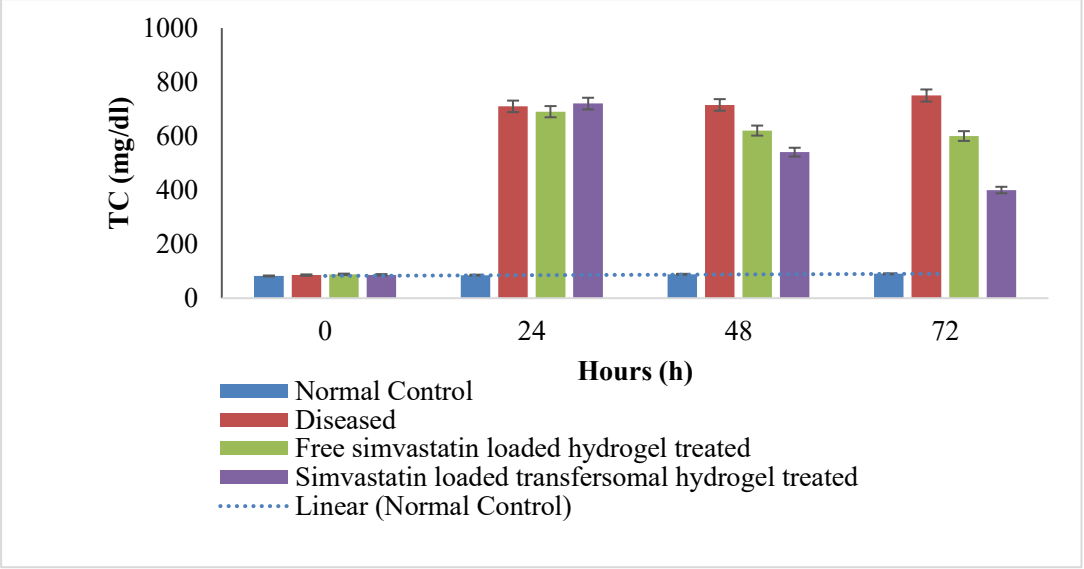

Figure 3.18. TC levels in different groups of hyperlipidemic rats.

Similarly, in TG graphs as shown in Figure 3.19, diseased control increases significantly from 50 mg/dl to 560 mg/dl in first 24 hours and to 750 mg/dl in 72 hours. Free simvastatin hydrogel and simvastatin loaded transfersomal hydrogel showed decrease in TG concentration i.e., decreases to 280 mg/dl and 220 mg/dl respectively after 72 hours.

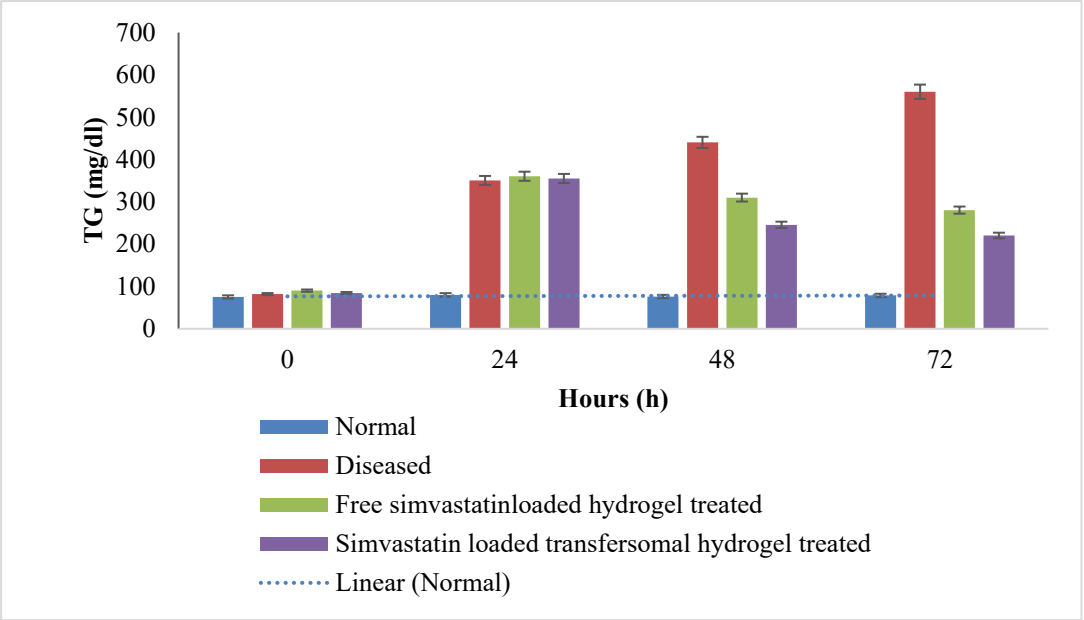

Figure 3.19. TG levels in different groups of hyperlipidemic rats.

In HDL/LDL ratio as shown in Figure 3.20, we can see that there is decrease in concentration while increase in free simvastatin hydrogel and simvastatin loaded transfersomal hydrogel.

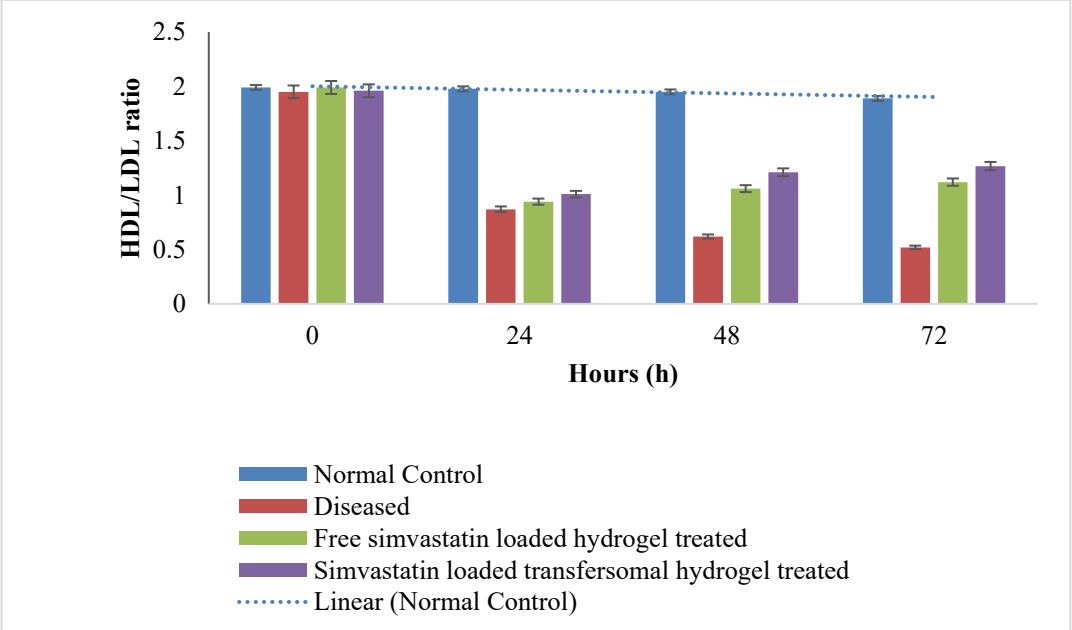

Figure 3.20. HDL/LDL ratio in different groups of hyperlipidemic rats.

In atherogenic index graph as shown in Figure 3.21, its value remained 0.2 in normal control after 72 hours. In simvastatin loaded transfersomal hydrogel, its value decreases to 0.518 after 72 hours compared to negative control (1.31) and free simvastatin hydrogel treated group (0.795).

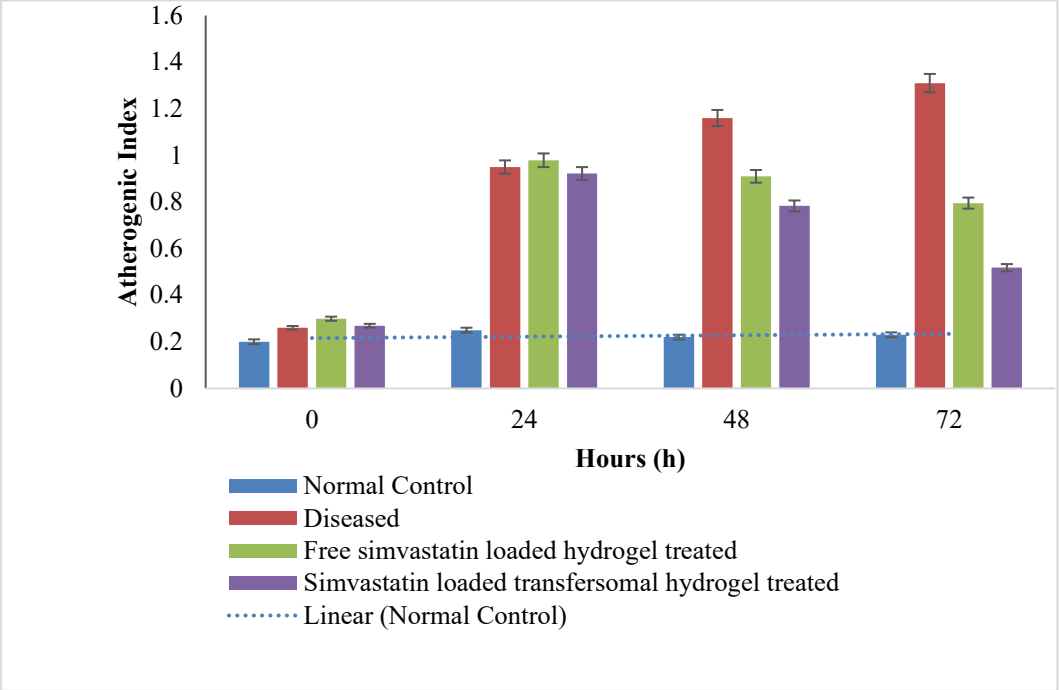

Figure 3.21. Atherogenic Index in different groups of hyperlipidemic rats.

CHAPTER 4

DISCUSSION

4. DISCUSSION

Simvastatin was loaded onto transfersomes and given trans-dermally to overcome the difficulties mentioned above. The transdermal route was chosen to shield the medications from GIT-related side effects that come with oral administration as well as to overcome dose related side effects of simvastatin that may include rhabdomyomas, increase of transaminases and provide sustained and enhanced effects. The purpose of study was to developed and enhance the sustained release system of antiatherosclerosis to prevent severity of atherosclerosis and avoid dose related and GIT related side effects of drugs (Anwer *et al.,* 2019).

There is direct relationship between phospholipids and vesicle size, as well as an inverse relationship between surfactant quantity and vesicle size in transfersomal formulation. The size of the vesicle increases as the concentration of phospholipids rises, and vice versa. There are two plausible explanations for this: first, as the concentration of phospholipon 90G rises, the viscosity of transferosomes rises, making it difficult for vesicles to disseminate in the system. Second, as the concentration of phospholipon 90G rises, multilamellar vesicles (MLVs) emerge, which are larger than uni-lamellar vesicles. When the amount of tween-80(surface active agent/edge activator) is increased, the size of vesicles decreases. This could be because there are less phospholipids available to form vesicles when the amount of tween-80 is increased. Another reason could be that as the amount of surfactant is raised further, mixed micelles form instead of vesicles, which are smaller in size (Khan *et al.,* 2015).

Entrapment efficiency is the percentage of drug accumulated inside the vesicular formulation. Estimation of entrapment efficiency is required because it aids in determining the formulation's ability to distribute drugs as well as the system's binding capacity. The concentration of phospholipids (phospholipon 90G) and the percentage of simvastatin encapsulated had a direct relationship; Entrapment efficiency rose as the number of phospholipon decreased.

Based on the size distribution of vesicles the homogeneity of nano-formulations is measured using the poly dispersity index method. The vesicles form a population that is either homogeneous or heterogeneous. The vesicles in a homogenous population have a PDI of less than 0.10, but the vesicles in a heterogeneous population have a PDI

of more than 0.3. The transfersomes in this study had a high degree of homogeneity and a narrow size distribution (PDI 0.273).

The zeta potential is the net charge on the surface of vesicles, which can alter the interaction of skin with vesicles as well as the formulation's physical stability. Higher values of negative or positive Zeta Potential will prohibit vesicles with the same charge from aggregating. Zeta potential was shown to be negative in transferosomes formulations. The optimized formulation had a value of -20.4 mV. The exposed negative charge on simvastatin transfersomes accounts for the negative zeta potential value. Because human skin has a positive ZP value, vesicles with a negative charge have a better skin contact. As a result, the formulation with a negative value will stick to the skin better.

Transferosomes' high membrane deformability allows them to squeeze through pores that are smaller than their diameter. Surfactant's capacity to interact with keratin and solubilize subcutaneous lipids and clear the passage through the skin layer is one explanation for better medication penetration.

To evaluate the identity of our drug, we performed analytical study to identify purity of our drug by following the ICH guidelines, a standard Analytical reported UV method was utilized to estimate simvastatin in transfersomes formulations. The result shows that all of the outcomes were within acceptable bounds (Tassaneeyakul *et al.,* 2006). Pre-formulation studies were conducted and excipients i.e., surfactant, and phospholipid (phospholipon 90G) were selected. Drug-excipient compatibility is an important pre-formulation study that examines the interactions between medications and excipients to minimize formulation difficulties and choose the best excipients for the final formulation. Incompatibility of a drug with its excipients can alter its therapeutic and physicochemical qualities, affecting the dosage form's stability, safety, and effectiveness (Thakur *et al.,* 2018). Consequently, FTIR is a crucial early study for determining interactions and developing the most effective and stable dose form. FTIR is used to check for drug-excipient interactions by comparing the primary peaks and looking for the disappearance/shifting of existing peaks as well as the appearance of new peaks, which are evidence of interactions. When comparing the FTIR spectra of a simvastatin to a reference, no new peaks develop and no substantial peak shifting occurs, indicating that the simvastatin and excipients are compatible. The FTIR analysis of simvastatin, phospholipon 90G, and tween 80 was compared to the FTIR

scan of the optimized transferosomes formulation, and it was confirmed that all of the major peaks were present in the final optimized formulation, indicating that the drug is not chemically interacting with any of the excipients (Nayak *et al.,* 2016). All the above were found in FTIR analysis of optimized simvastatin loaded transferosomes.

Then we formed our final dosage form that is simvastatin loaded transfersomal hydrogel. The addition of transfersomal dispersion gave the hydrogel a milky white look. The hydrogel's compatibility for skin application was determined by its pH of 5.6, which is within the permissible pH range of 5.5 to 7.

The viscosity of the hydrogel grew when the carbopol 934 concentration was increased, and the viscosity of the hydrogel dropped as the shear rate was raised to 700, confirming the hydrogel's shear thinning tendency. The optimized hydrogel's simvastatin content was about 93.1%, indicating that the simvastatin loaded transfersomal hydrogel was spread uniformly throughout the hydrogel. Spread ability increases as viscosity of simvastatin loaded transfersomal hydrogel decreases, therefore less viscous hydrogels are more spreadable.

The optimized simvastatin loaded transdermal hydrogel primary skin irritation studies were performed to (Porfire *et al*., 2017) confirm the safety of simvastatin loaded transdermal hydrogel. Formalin control showed the edema and erythema and changed in the skin structure indicating value of 1 and 2 while simvastatin loaded transfersomal hydrogel have score of 0 indicating no reaction on skin. Histopathology of skin showed no damage to the epidermis in the optimized gel no infiltration of T-cells, confirming the safety of using simvastatin loaded transfersomal hydrogel.

In Vitro drug release profile of pure drug and transfersomes was studied by using dialysis bag method. The transfersomes loaded transfersomal hydrogel shows high cumulative release as compared to free simvastatin loaded hydrogel. As all result which studied on pH 7.4 shows that our formulation is quite stable and produce sustained effects for long period of time. Followed by Korsmeyer-peppas kinetic model, which shows that our formulation follows Fick’s diffusion (Qin *et al.,* 2011).

Diffusion studies were performed on Franz diffusion cell on simvastatin loaded transfersomal hydrogel and free simvastatin loaded hydrogel. These studies showed that simvastatin loaded transfersomal hydrogel have higher permeation as compared to the

free simvastatin loaded hydrogel which may be due to the flexible nature of transfersomal membrane which allows squeezing of transfersomes through the pores. other mechanism to support the behavior of simvastatin loaded transfersomal hydrogel is acting of transfersomes as penetration enhancer resulting in the increase of overall membrane fluidity. This mechanism to support the behavior of simvastatin loaded transfersomal hydrogel in diffusion study is the action of transfersomes as drug carrier system, which carries the entrapped simvastatin across the skin under action of transcutaneous hydration gradient (Qin *et al.,* 2011).

Because of its rapid inhibition of the lipoprotein lipase enzyme and lack of strong toxic effects, poloxamer 407 causes hyperlipidemia in rats quickly and causes substantial hyperlipidemia within a few hours. LDL, HDL, TC, and TG metabolism are all affected by poloxamer 407 in a variety of ways, including by inhibiting lipoprotein metabolism.

The hydrolysis of TG by lipase and the body's indirect manufacture of cholesterol. The poloxamer injection considerably elevated the levels of cholesterol in a11 groups during the first 24 hours. remarkedly in all the groups. Similar was the case with vLDL as shown in table 2. In case of HDL-c, the level of HDL decreases sharply within first 24 hours after the poloxamer 407 injection. The normal group didn’t show any significant change in the levels of cholesterol, TG, HDL, and vLDL which proves the hyperlipidemic action of poloxamer 407. The transdermal administration of free Simvastatin and simvastatin loaded transfersomal hydrogel decreased the level of hyperlipidemia which also decreased the atherogenic index in 72 hours study.

From the graph, comparing the atherogenic index between the diseased control group, Free simvastatin hydrogel treated group and simvastatin loaded transfersomal hydrogel treated group, it is shown in results above showed that the atherogenic index was lowered in simvastatin loaded transfersomal hydrogel treated group of Poloxamer 407 induced hyperlipidemia. Increased cholesterol level and triglyceride level with decreased high-density lipoproteins in serum may give rise to atherosclerotic plaque. simvastatin loaded transfersomal hydrogel reduced the TG and TC levels significantly. LDL levels were significantly decreased by simvastatin loaded transfersomal hydrogel therefore results imply that simvastatin loaded transfersomal hydrogel provides advantage by decreasing lipid profile.

CONCLUSIONS

- Simvastatin was successfully loaded in transfersomes with optimized particle size, PDI and zeta potential.
- Characterization of simvastatin transfersomes and hydrogel showed significant results.
- *Ex vivo* studies indicated improved penetration of prepared formulation through transdermal layers.
- Stability studies showed that prepared simvastatin loaded transfersomes and simvastatin loaded transfersomal hydrogel are stable up to 6 months.
- *In vivo* animal studies have shown marked decrease in atherogenic index, vLDL, TG and TC values while increase in HDL value.

FUTURE PROSPECTIVES

- Extrapolation of theranostic could provide more information regarding the efficacy and safety of dosage form.
- Large scale clinical investigations are required to demonstrate its effects on human population.
- Different dosage forms of simvastatin based transdermal hydrogel can be compared with oral dosage form to evaluate efficacy, safety, and systemic release.
- Pharmacokinetic studies can be performed to evaluate bioavailability, half-life, area under curve.
- Further studies are required to access the feasibility of this formulation at industrial scale.

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Annexure I: Approval from Bioethics Committee

قائداعظم یونیورسٹی
QUAID-I-AZAM UNIVERSITY
Faculty of Biological Sciences
Bioethics Committee

No. #BEC-FBS-QAU2022-442 Dated: 18-10-2022

Ms. Syeda Ayesha Mazhar
C/O Dr. Kifayat Ullah Shah,
Department of Pharmacy,
Faculty of Biological Sciences,
Quaid-i-Azam University, Islamabad
45320, Pakistan

Subject: - **"Development and Evaluation of Simvastatin Loaded Transfersomal Hydrogel for the Management of Atherosclerosis."**

Dear Ms. Syeda Ayesha Mazhar,

We wish to inform you that your subject research study has been reviewed and is hereby granted approval for implementation by Bio-Ethical Committee (BEC) of Quaid-i-Azam University, Your study has been assigned protocol **#BEC-FBS-QAU2022-442.**

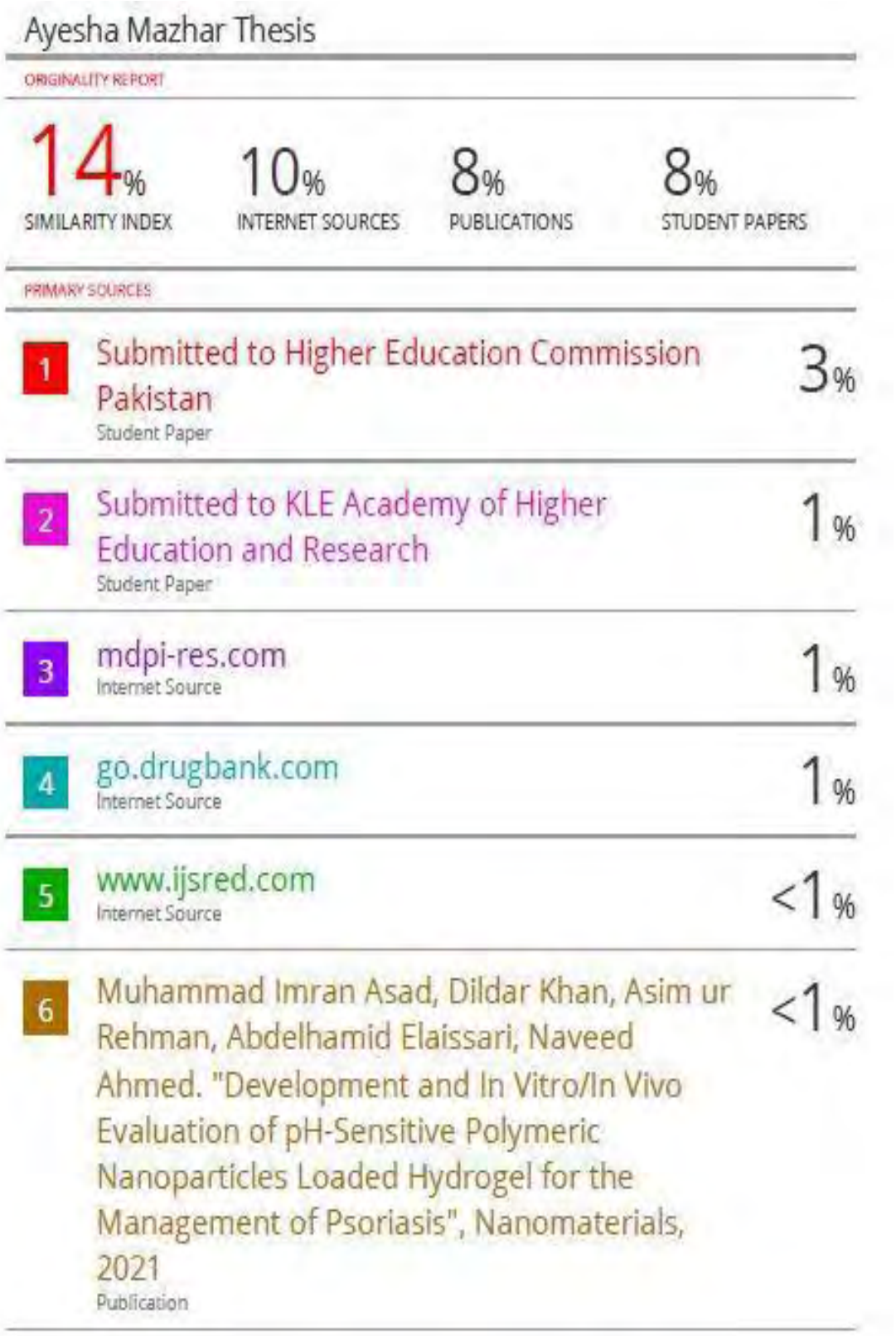
While the study is in progress, please inform us of any adverse events or new, relevant information about risks associated with the research. In case changes have to be made to the study procedure, the informed consent from and or informed consent process, the BEC must review and approve any of these changes prior to implementation.

Sincerely,

Prof. Dr. Sarwat Jahan
Department of Zoology

cc:
Dean, F.B.S

Annexure II: Turnitin Similarity Index Report



Ayesha Mazhar Thesis

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