An anti – glaucomatous protein - based in-situ nano emulgel with considerable stability without surfactants and co-surfactants

Research thesis submitted to Thapar Institute of Engineering and Technology (TIET) in partial fulfillment for the award of the degree of "Masters of Science In Biochemistry"

Submitted by

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(Deemed to be University)

DECLARATION

I hereby declare that the dissertation work entitled **"An anti – glaucomatous protein - based in-situ nano emulgel with considerable stability without surfactants and co-surfactants"** is based on original research executed at National Institute of Pharmaceutical Research and Education,Hyderabad under the supervision of Dr. Pankaj Kumar Singh,Assistant Professor, NIPER Hyderabad. I affirm and assure this entire research work is original and well preserved with authenticity. Moreover, it was never earlier submitted in part or full, for any other professional degree or Diploma to any Institution in India and abroad.

Harshita Kaur

(July29,2023)

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CERTIFICATE

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राष्ट्रीय औषधीय शिक्षा एवं अनुसंधान संस्थान
औषधीय विभाग, रसायन एवं उर्वरक मंत्रालय, भारत सरकार
National Institute of Pharmaceutical Education and Research
Department of Pharmaceuticals, Ministry of Chemicals & Fertilizers, Govt. of India

Date: 15/07/2023

TO WHOMSOEVER IT MAY CONCERN

CERTIFICATE

This is to certify that Harshita Kaur, studying Masters in Biochemistry, in Thapar Institute of Engineering and Technology, Patiala, Punjab has successfully completed the project internship in department of Pharmaceutics, NIPER Hyderabad during the period from 19/01/2023 to 13/07/2023.

During the above said period, her conduct was found to be good. We wish her all the best for her future endeavour.

15/7/2023

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I am blessed that my destination to get featured is Thapar Institute of Engineering and Technology, Patiala. I was wandering through and keeping track of unapproachable "stars"; however, I was puffed with a storm of regrets extending through my nerves within!

Then, I got shelter and, all the privileges of my life on this campus. The life I had here, I had never even imagined to have. I had never thought, once, that I would be a student of this blessed home.

This institute gave me constellations to walk upon and align myself to aimed stars. I am not only dedicated to being successful for my family, and myself but utmost for my mentors – Dr. Diptiman Choudhury, Dr.Neloy Chakroborty, Dr. Mily Bhattacharya, Dr. Niranjan Das, and all the admirable faculty of Thapar Institute of Engineering and Technology who invested their knowledge, precious time within me and this institute personalized wings for me supported by dedication, honesty, passion and orientation to pursue PhD. in abroad.

Dr. Diptiman Choudhury, my father figure supported me and had faith in me when I lost faith in myself. He blessed me with more than what I deserve. He applied the required torque to my not-so-worthy life and muffled passion to follow knowledge. He enlightened my life with hopes and fabricated a capable version of me. He invested his priceless efforts to pull me out of the darkness I used to live in!

He had sown seeds of curiosity, boldness, and the value of being organized in life. I am blessed to find a shade under his hands full of blessings for my research future.

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Dr.Mily Bhattacharya is incredibly different in each way. She is unbelievably well-versed in her subject that her every single subject discussion swayed me with it and I loved to slowly unravel within the world of "Protein Chemistry". For my entire life, I will learn from never explained concepts she cleared. Her recorded classes are in touch with my heart!

She is calm-collected and a manifestation of professionalism. I am blessed to be a student of such a lovely soul. "Kabhi Kabhi apne spnon ko dhup lagwani chahiye aur pani dena chahiye", ma'am I wrote this in a notebook of my heart. The way you struggled to be in this field has inspired me always. Thank you so much, ma'am for being there. I wish god to bless you with all his hands full for the research project you are investing yourself in!

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I am extremely blessed for the eternal love and support from my lovely parents, angel-sweet sister, and my fiance, Mohit Taneja. I promise them not to take their love for granted.

I extend my thanks to my supervisor for this project Dr. Pankaj Kumar Singh, NIPER-H. I learned a lot in his lab!

For this project goat eyes were sacrificed to accomplish ex-vivo permeation studies and confocal imaging which was the hardest portion of it pursue for me. I apologize to god and thank those innocent goats whose organs were taken for this project.

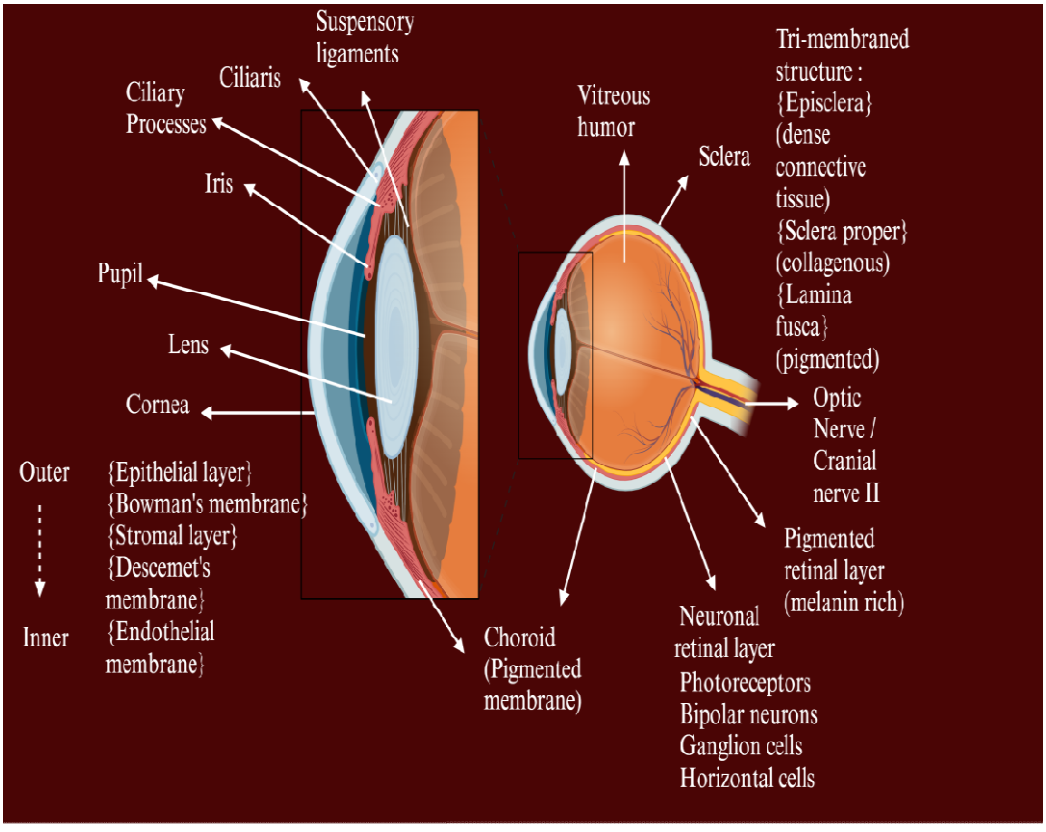

Figure : 1 The sagittal view of human eye (Created with BioRender.com)

Figure : 2 Research assignment motivation

TABLE OF CONTENTS

ABBREVIATIONS :

HPLC : High Performance Liquid Chromatography

DSC : Differential scanning calorimetry

TGA : Thermogravimetric analysis

NMR : Nuclear Magnetic Resonance

PXRD : Powder X-Ray Diffraction

PDI : Polydispersity Index

BSA : Bovine Serum Albumin

STF : Simulated Tear Fluid

List of tables and figures :

TABLES :

FIGURES :

GRAPHS :

Pre-formulation analytical research of edaravone :

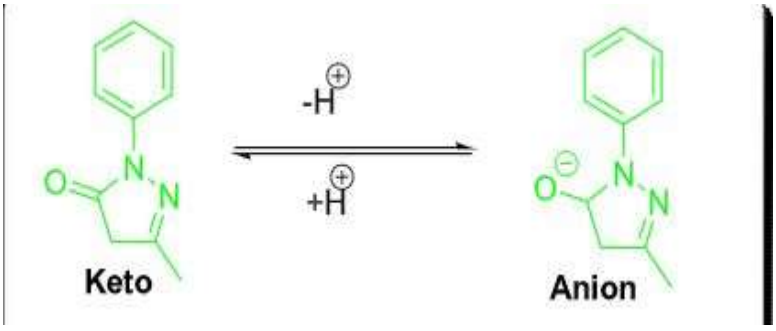

Figure 3 : Chemical structure of edaravone (created by chemdraw)

--→ Nuclear Magnetic Resonance Spectroscopy : (Bruker Ascend 500)

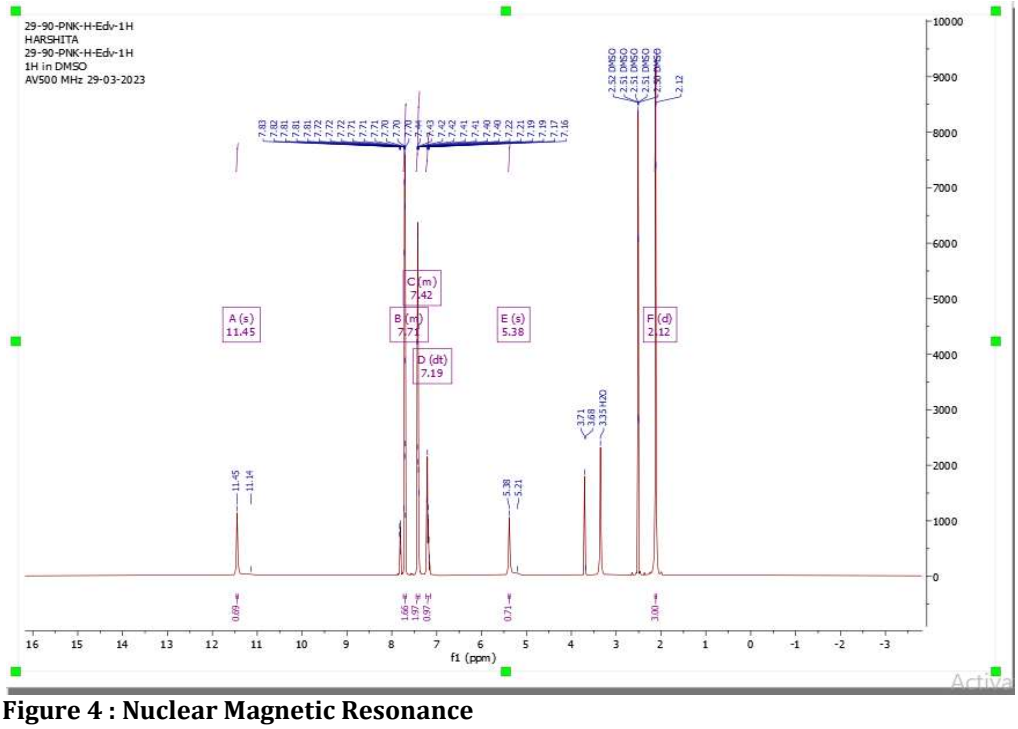

Figure 4 : Nuclear Magnetic Resonance

CDCL 3 1H NMR (CDCL 3): (CH2 3.1), (CH3 2.2), (5H, Ar-H, 7-8)

--→ <u>Powder X-ray Diffraction</u> :

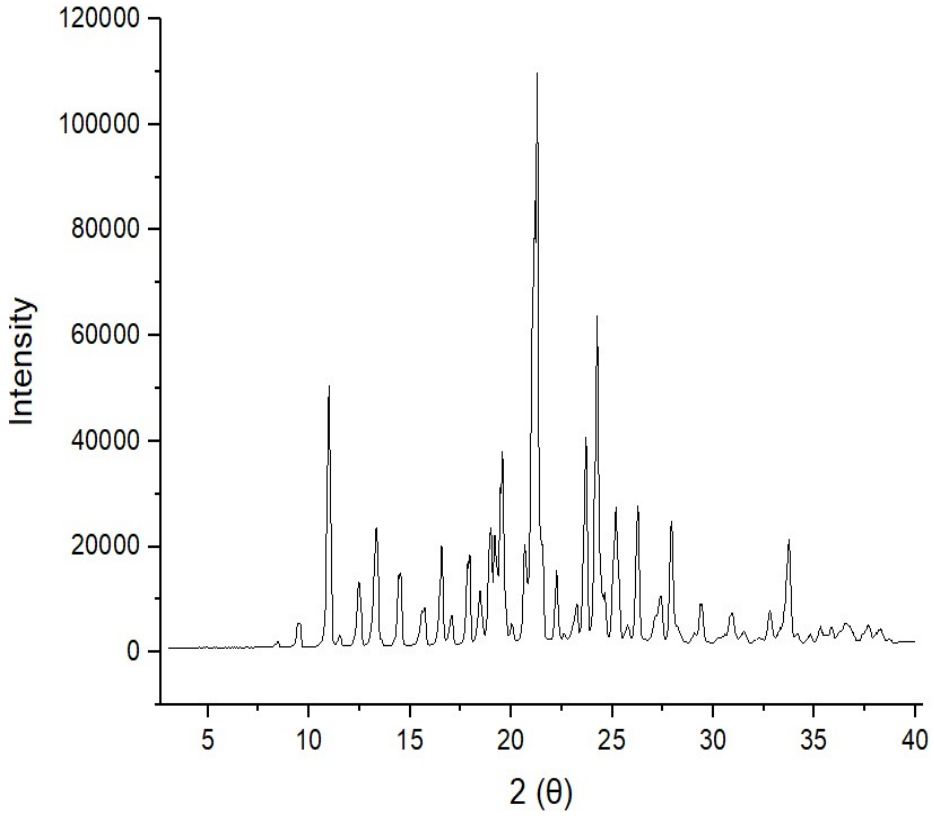

Figure 5 : Powder X-ray Diffraction

--→ **Differential Scanning Calorimetry : (DSC2500)**

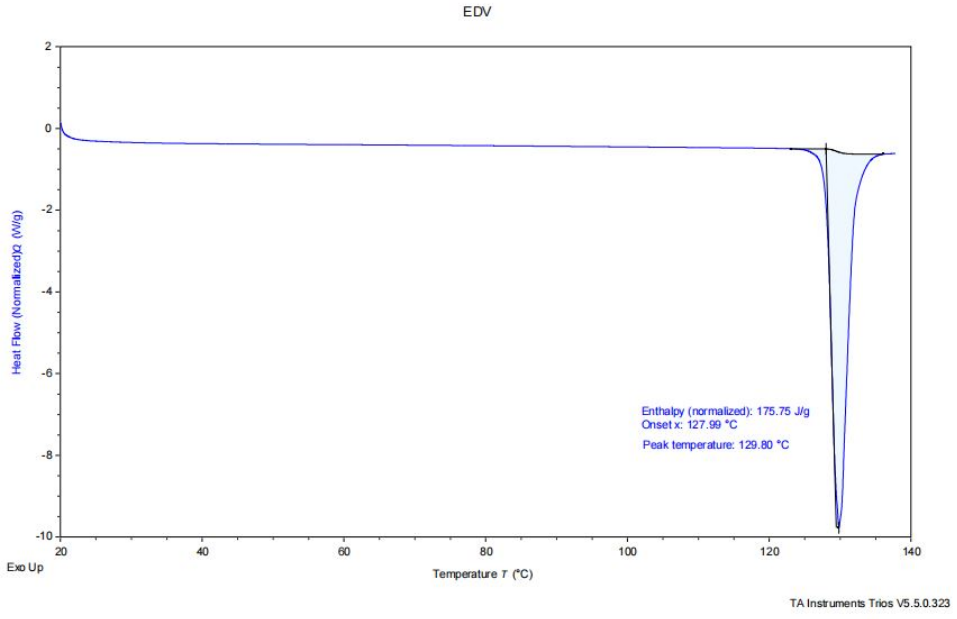

Figure 6 : Differential Scanning Calorimetry

--→ **Thermogravimetric analysis : (TGA500)**

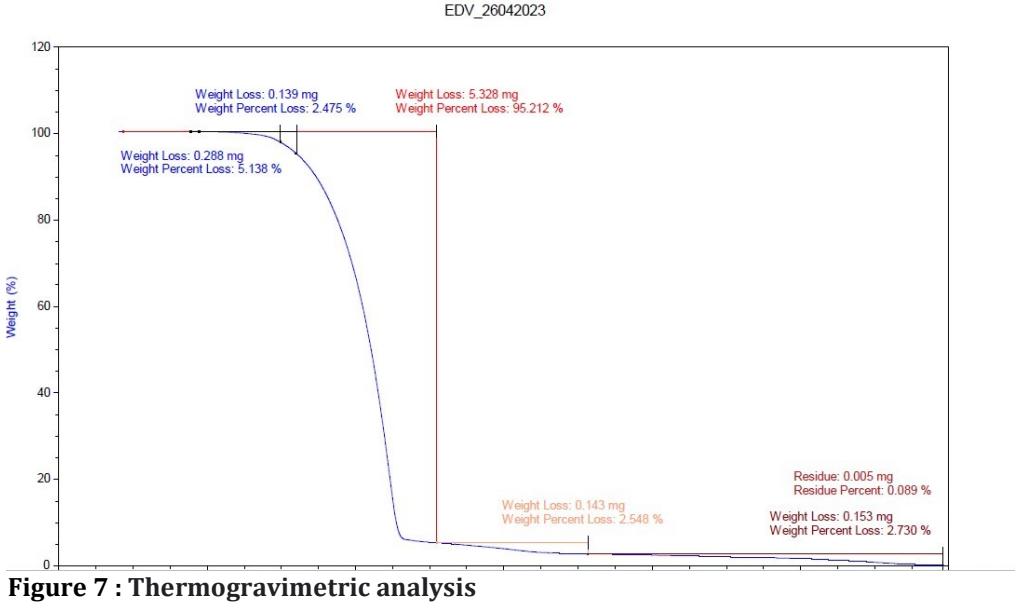

Figure 7 : Thermogravimetric analysis

HYPOTHESIS :

Neuroprotective effect of edaravone in experimental glaucoma model in rats: a immunofluorescence and biochemical analysis

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administration at the start of the glaucoma process. Statistically significant lower NO levels were determined in the glaucoma group comparing treatment groups (Bonferroni, $P<0.05$). MDA levels were found to be highest in untreated glaucoma group, TAC levels were found to be lower in the glaucoma induction groups than the control group (Bonferroni, $P<0.05$).

• CONCLUSION: Systemic administration of edaravone in experimental glaucoma showed potent neuroprotective activity. The role of oxidative stress causing RGC

Therapeutic potential of curcumin in eye diseases

Dorota M Radomska-Leśniewska [1], Anna Osiecka-Iwan [1], Anna Hyc [1], Agata Góźdź [1], Anna M Dąbrowska [2], Piotr Skopiński [1]

Affiliations + expand

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Abstract

Curcumin (diferuloylmethane) derived from the rhizome of Curcuma longa L. has been used for thousands of years in traditional Chinese medicine and Ayurvedic medicine in Asian countries to treat liver diseases, rheumatoid diseases, diabetes, atherosclerosis, infectious diseases and cancer. It exhibits a wide range of pharmacological properties, which include antioxidant, anti-inflammatory, antimutagenic, antimicrobial and anticancer activity. Herein the mechanisms of curcumin impact on oxidative stress, angiogenesis and inflammatory processes are described indicating that curcumin use may inhibit those pathological conditions and restore body homeostasis. Its effectiveness was also proved for major eye diseases. In this review, the influence of curcumin on eye diseases, such as glaucoma, cataract, age-related macular degeneration, diabetic retinopathy, corneal neovascularization, corneal wound healing, dry eye disease, conjunctivitis, pterygium, anterior uveitis are reported. The analysis of a number of clinical and preclinical investigations indicates that curcumin may be used as a therapeutic agent in the treatment of various eye disorders.

Keywords: age-related macular degeneration (AMD); angiogenesis; cataract; conjunctivitis; curcumin; diabetic retinopathy; eye disease; glaucoma; reactive oxygen species (ROS).

Abstract :

Continuous vision impairment in glaucoma due to persistant death of retinal ganglions can lead to permanent blindness. Delivery of drug to particular parts of the anterior or posterior segment has been a major challenge due to various protective barriers and

elimination mechanisms associated with the unique anatomical and physiological nature of the ocular system. Drug administration to the eye by conventional delivery systems results in poor ocular bioavailability.

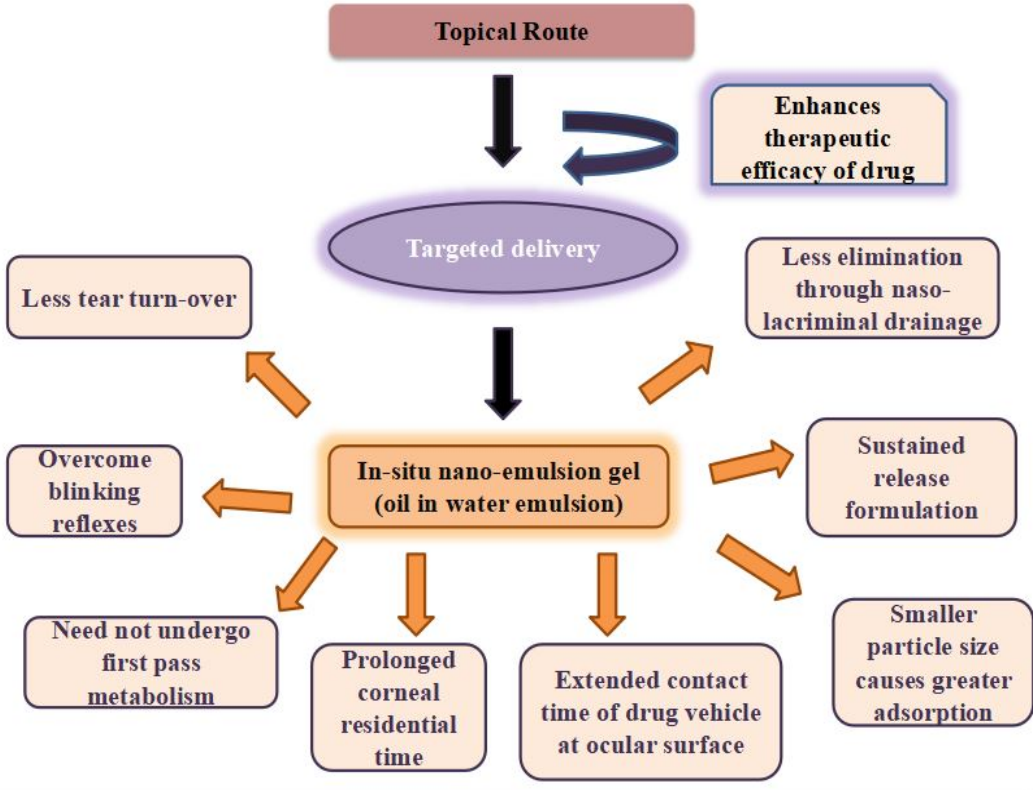


Figure 8 : Research Territory

Introducion :

The organ of sight: an eye is structured relatively delicate with a distinguishable network of organization. These pairs of eyes complement each other to be more efficient to perform their predominant function which is to impart vision. It comprises distinctively held each layer that corresponds to the intrinsic biochemical nexus. Moreover, each part of this systematically evolved organ contributes to a respective functionalization which all – together performs incredibly systematic. There is indeed a requirement to establish a more efficient topical ophthalmic drug administration system to deliver potent pharmaceutical preparations against chronic eye disorders. Drug compounded in-situ gel is an influential approach to optimize a formulation that would undeniably over – power numerous disadvantages of conventional ophthalmic therapeutic preparations. These are stimulated with a sort of mechanism which remain in free–flowing liquid state of matter before instillation into an eye, howsoever,

transform it's state to a viscous gel after it gets incorporated with eye – structure. The gelation time (sol–gel reversible transition) depends upon the components of the formulation. This sustained-release variant of pharmaceutical preparation remarkably considers lesser pre–corneal residence time (RT) as a demerit of various medicated eye-solutions which is feasible to get enhanced via thixotropic in–situ gels and reduces lacrimal secretions. As pre – corneal residence time is interdependent and directly proportional to tear film thickness present at the ocular surface. Blinking reflexes and eliminations through nasolacrimal drainage are reduced which keeps it less – tedious to administer a topical ophthalmic dose. In-situ gels are more substantial and efficient as there is evident penetration of the drug and higher pharmacological efficacy in certain research specimens.

1. **Materials :** Different constituents of nanoemulsion were indented from respective manufacturing units to formulate a potent therapeutic pharmaceutical preparation. Lupin Limited, Pune, Maharashtra, India assisted with Radicava; a solid crystalline drug to repurpose. Proprietary unani medicinal kalonji oil which comprises over 58% of essential amino acids (glutamic acid, aspartic acid, arginine, leucine, and glycine), inorganic phosphorus, iron, and bioactive thymoquinone compound was purchased from Mohammedia Products, Telangana state, India. Bovine serum albumin(BSA) which was significant to act as an emulsifier in nanoemulsion was bought from Avra, Hyderabad, Telangana, India. There were two gradients of the same pluronic gelling – agents pluronic 188 and pluronic 127 were put into application to formulate in–situ emulsion-based gel preparation and these were fetched from Himedia, Thane(West), Maharashtra, India, and Sigma-Aldrich, Banglore, India.

2. **Experimental Methodology :**

2.1 Screening studies of oil :

The most appropriate oil with concern to protein-stabilized nanoemulsion was recognized followed by a drug-solubility test. There were ten different oil components (Table 1) considered for drug-solubility experimentation taking ophthalmic compatibility in context to toxicity profile elucidated in literature and therapeutic potential into consideration with utmost care. The entire formulation was prepared with kalonji oil. (Figure 9)

Conscientiously, a set – protocol was followed to select the most relevant oil for nanoemulsion. 35 mg (excess drug) was added to 500 mg ± 5mg oil(w/w) withdrawn in 2 mL eppendorf which remain covered with aluminum foil to avoid photon perturbation and incubated for entire 72 hours in a shaker incubator at a speed of 100 revolutions/minute at 36°C which corresponds to ocular surface temperature.

Enlisted oily – phase components for experimentation	Respective categorical group
Oleic acid	Mono-saturated omega-9 fatty acid
Soyabean	Vegetable oil
Labrafac	Vegetable oil
Linseed	Vegetable oil
Capmul	Hydrogenated vegetable oil
Capryol 90	Absorption enhancer
Kalonji	Vegetable oil
Castor	Vegetable oil
Olive	Refined oil
Corn	Refined vegetable oil

Table 1 : Variable Components taken to perform solubility experimentation

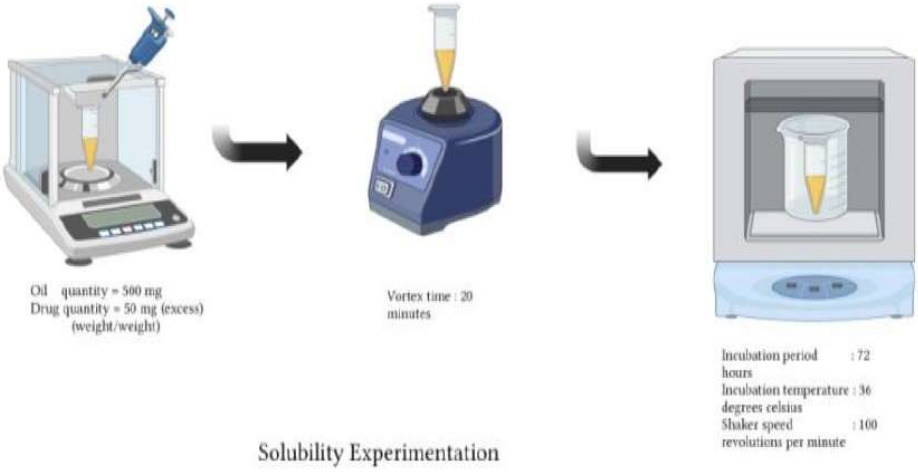


Figure 9: Solubility Experimentation

2.2 HPLC Method Development

High Performance Liquid Chromatography (HPLC) is a highly sophisticated analytical equipment which quantitatively isolates numerous components in entire pharmaceutical product composition.

The principle of chromatography:

When each component dissolved in the mobile phase passes through the stationary phase, the size and strength of each component's interaction with the stationary phase (adsorption, distribution, exclusion, affinity) are different. Therefore, the residence time of these components in the stationary phase is different, flowing out from the stationary phase at different times one after another. The essence of chromatography is

a method of separation and analysis. Liquid chromatography (LC) refers to when liquid is used as the mobile phase. The HPLC method was developed at Shimadzu's LabSolutions software which was ingeniously – tailored and programmed to pursue methodological systematic research.

During, pre – formulation investigations the (λ_{max}) of drug was achieved through Jasco UV-Vis Spectrophotometer with Spectra Manager software. The HPLC method was developed against (λ_{max}) determined. Consequently, the essential parameters that were held constant are curated in (Table 2)

Column Type	C18
Column Length	250 mm
Detector	UV+PDA
Wavelength ((λ_{max})	244 nm
Mobile Phase	Millipore water : Acetonitrile
Solvent B concerntration	60
Temperature	Ambient
Pressure	5000 psi
Flow rate	1mL /minute
Injection volume	10 µL
Run- time	6 minutes
Retension time	3.2 minutes

Table 2 : Chromatographic conditions

2.3 Preparation of Protein-stabilized nano-emulsion

The pharmaceutical preparation mandatorily demands to adhere to the entire established protocol as proteins should always be handled with the utmost care due to protein – sensitivity towards external stimuli. There were several experimental trials held to confirm and validate the most compatible composition taking critical attribute: phase separation, polydispersity index, zeta – size, and zeta potential into consideration. The arithmetic mean values must correspond to standard values for subsequent execution.

Preparation of protein solution :

Initially, a 1% (weight/weight) bovine serum albumin solution was prepared to stabilize kalonji oil in phosphate buffer saline nano-emulsion loaded with RADICAVA® (edaravone) given that bovine serum albumin has an amphiphilic conformation that holds both hydrophilic and hydrophobic domains within a folded – globular structure. To safeguard protein strand denaturation, bovine serum albumin (BSA) must be processed carefully and stored at 4 degrees celsius.

1000 mg of accurately weighed protein was added slowly to 100 ml miilipore water placed upon magnetic heat stirrer plate at 50°c at the rate of 550 revolutions per minute

for 45 minutes for "*activation of protein"* . Thereupon, the bovine serum albumin solution was kept for overnight stirring at room temperature for entire incorporation of protein into solvent . It is evident that water's flux potential on protein surface makes it sufficiently dynamic to be biologically active. This has been investigated that for higher biological impact soluble proteins predominantly require water molecules to extend them so forth to absolutely cover protein structures . Immediately , after activation of bovine serum albumin in a solution ; the activated solution was kept at 4 degrees for complete 2 hours for "*peptide – hydration"*.Moreover, storage direction for prepared protein solution is also refrigeration at 4 degrees celsius .Researchers determined that biological behavior of a protein molecule corresponds to temperature dependent to a higher extent and discovered that when proteins are quiescent at temperatures below 30°C, the molecules rotate around their own axes due to water movement on the protein surface. However, beyond 30°C, the water molecules begin to undergo translational diffusion while continuing to rotate. This is the temperature at which proteins become active, and those studying them believe that the ability of water molecules to "exercise" on the surface of proteins allows them to function.

The scientists generated these conclusions through the combination of neutron scattering with molecular dynamics simulations. The neutron scattering technique provides precise data on atom and molecule mobility and local configurations in materials.

Loading of Radicava to kalonji oil :

The 400 µL kalonji oil was pipetted out into amber coloured 1.5 mL eppendorf and 50 mg drug (Radicava) was precisely weighed was soon after added to oil . The drug–oil mixture was vortexed for 20 minutes and then kept in shaker incubator under appropriate physicochemical for 72 hours at a speed of 100 revolutions/minute at 36°C which is corneal surface temperature.

Preparation of nano – emulsion :

After experimentation trials with (0.5%,1%,2%,3%,4%) out of which 1% was optimized which means 9 mL and 600 µL of chilled bovine serum albumin (BSA) solution were withdrawn into an aluminum foil-wrapped beaker and homogenized (Polytron PT 2500 E) at 1350 rpm for 20 minutes, whilst 400 µL of kalonji oil mixture was added dropwise to the protein solution. After the addition of 10 mL, the concoction was left undisturbed so as to be get homogenized thoroughly under homogenizer. The obtained drug loaded nanoemulsion was preserved at 4°C.

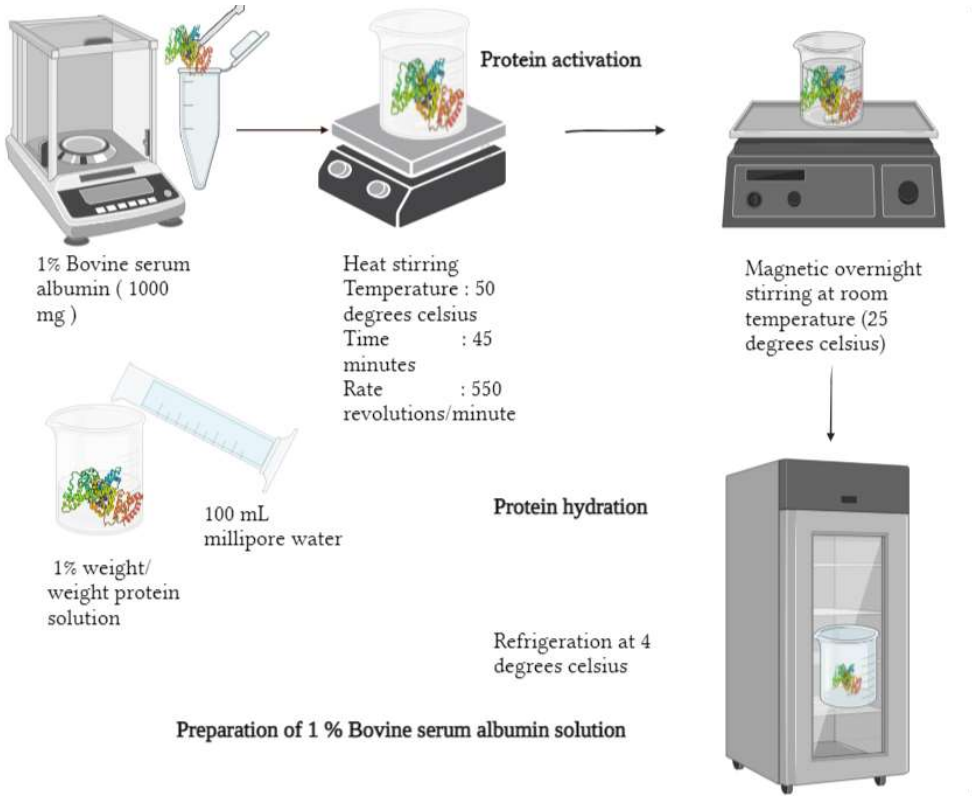


Figure 10 : Preparation 1 % Bovine serum albumin solution

Thermo- responsive reaction transforms nano-emulsion to in-situ emulsion gel :

After optimization trials executed against stability and expected thermoresponsive effect the combination of two distinct grades of pluronics; non-ionic tri-block co-polymers with a hydrophobic center which exhibit self-assembly and undulation upon elevated temperatures. 167 mg of Poloxomer (F188) and 1000 mg of Poloxomer (F127/F404) were added and mixed with a spatula in an ice bath until a cohesive and standardised fluid solution emerged. At 4 °C, the in-situ gel formulation was liquified however at 34 °C a consistent gel was formed. The gelation temperature was found to be 1 minute.

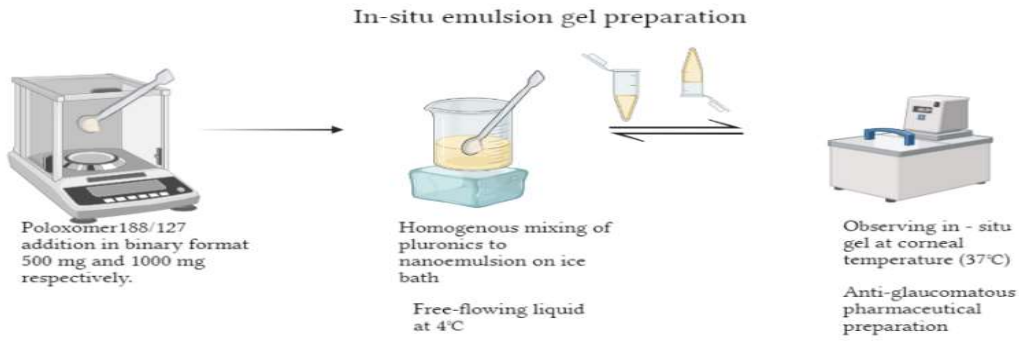


Figure 11 : In-situ emulsion gel preparation

Chemical Interaction of Pluronic with Formulation at Variable Temperatures :

Poloxomer consists of hydrophobic polypropylene oxide center sandwiched between hydrophilic ethylene oxide conjugates which reveal self-assembly and undulation upon increasing the temperature. Polypropylene oxide tends to become less – soluble in water when there is rise in temperature and the chains form aggregate to reduce surface contact of polypropylene oxide with water .

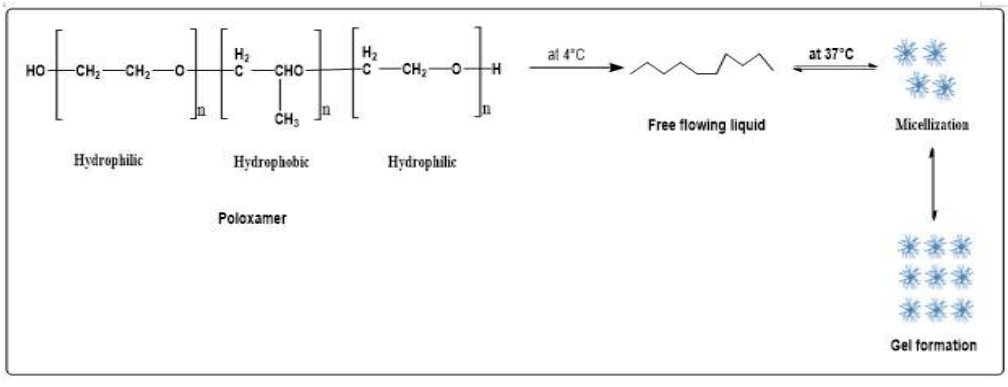


Figure 12 : Poloxamer Chemistry (Created by chemdraw)

Encapsulation of *Radicava*® loaded into drug vehicle :

The drug content experimentation strives to calculate drug potency for therapeutic effect. Separate approaches were followed for nanoemulsion and in–situ emulsion gel. This investigation is predominant to proceed further with in-vitro and ex-vivo drug percentage release studies and calculate cumulative drug migration kinetics. The entrapment efficiency of drug is methodologically driven by (total drug added – drug which remained untrapped) divided by the total loaded drug to vehicle.

4. Drug content percentage ratio estimation for nano-emulsion :

The emulsion was diluted with acetonitrile, which was a mobile phase (organic) for high–performance liquid chromatography intends to lessen interferences from other components present in a formulation which are therefore, able to alter the analysis.

100 *µL of nano-emulsion (performed in triplicates; n=3) was withdrawn in an 2* mL eppendorf *from a 10* mL formulation batch which consists of 5 mg drug per mL bovine – serum stabilized nano-emulsion. *100* µL pharmaceutical preparation was diluted with 900 µL acetonitrile and was vortexed for 10 minutes to encourage homogenization throughout. Afterwards, it was filtered from 0.22µm (Polyvinylidene difluoride PVDF ,Supertek) non – sterile double membraned syringe filter and preserved for analytical measurement (high – pressure liquid chromatography) .

Drug content percentage ratio estimation for in-situ emulsion gel :

The gel preparation demands multifold dilutions for the purpose to decrease solute concerntration in formulation. Higher values of concerntration impede analytical measurement. There is a considerable probability of error occurrence due to high pressure in precipitated pump establishes dynamically lower flow rate of mobile phase through column and as a consequence analytical measurement comes to halt. The samples must be well – prepared without negligence anticipating scientific interferences. 109.3 mg (weight/volume) which is equivalent to two drops of in-situ gel was diluted with 5 mL acetonitrile and was vortexed for 10 minutes or even more until and unless it becomes homogenous and was refrigerated at 4°c for another 20 minutes as it is in poloxomer based thermoresponsive in-situ gel. Afterwards, it was filtered from 0.22µm (Polyvinylidene difluoride PVDF ,Supertek) non – sterile double membraned syringe filter and preserved for analytical measurement in amber colored HPLC vials at 4℃.

5. Characterization and evaluation of bovine serum albumin (BSA) stabilized nanoemulsion :

The protein stabilized nanoemulsion and emulsion in – situ gel were prepared and characterized for physicochemical properties using analytical tools. The prepared formulation was screened for physical observation,globule size,and polydispersity index (PDI),pH and surface morphological behavior.The prepared emulsion gel was screened for physical observation,Rheological behavior,spreadability behavior, pH and surface behavior.

5.1 Physical observation :

The protein stabilized nanoemulsion and and in- situ emulsion gel were evaluated by physical appearance in colour,turbidity and precipitation.

5.2 Globules size and PDI :

The globule size and PDI of protein stabilized nanoemulsion were determined using a particle size analyser (Malvern-Nano ZS,UK) and the principle involved is the operation is Dynamic light scattering (DLS). Firstly, 200µL nano-emulsion was diluted with 800 µL Millipore water to ensure globules with lesser density in order to prevent inter-globule interactions,then simultaneously poured into a zeta cuvette which is operated at 90° scattering angle and the cell unit was maintained at 25°C. The refractive indices were most essential parameter for determination were 1.33 and 1.470 for Millipore water (dispersant)and kalonji *(Nigella sativa)* oil globules respectively. All the measurements were extracted in triplicate series and reported as mean ± SD. (Figure 14 and figure 15)

5.3 Zeta Potential : The zeta potential for nanoemulsion were determined using a particle size analyser (Malvern-Nano ZS,UK).

5.4 pH of nano – emulsion and in-situ emulsion gel formulation :

The pH of protein stabilized nanoemulsion was determined using pH meter (Mettler Toledo,US).The pH meter probe was dipped inside the nanoemulsion and pH was measured. The pH of in-situ emulsion gel was determined by mixing one gram of gel with 100 mL of distilled water and kept undisturbed for couple of hours. Afterwards, measurements were performed in triplicate series and reported as ± SD.

5.5 Rheological studies :

Rheological properties and behavioral changes of in-situ emulsion gel pharmaceutical preparation using Modular Compact Rheometer (MCR 102, Anton-Paar Pvt. Ltd. Ostfildern, and Germany) that is equipped with peltier temperature control at 25°C.All the measurements were performed using a parallel plate spindle with a diameter of (25pp).An adequate amount of sample was applied onto base plate, with a sample gap of 1mm and an instrument was run for rheological studieswith respect to time as well as amplitude sweep test were performed to determine dynamic modulus (G') and loss (G'') modulus as a function of torque.(Figure 16)

5.6 Spreadability test :

The spreadibility test of in-situ emulsion gel was performed using a TAXT-Plus texture analyzer (New Delhi,India) within which a male rotational probe was installed which was further calibrated for its height before sample analysis .The formulation samples were loaded onto female cone – shaped receiver. However, the samples must be loaded onto clean conical surface carefully to avoid air bubbles in samples which can cause discrepancy in results to be reported. The analysis measurements were performed at 25°C which is room temperature. (Figure 18)

5.7 Osmolarity estimation :

It is mandate and clinically significant to measure osmolarity that is number of solute particles present in a formulation. Ideally, osmolarity of an ophthalamic solution must maintain ocular isotonicity. The osmolarity measurement (Gonotec, OSMOMAT 3000) was performed with minimum of 50 µL sample was withdrawn in an 1.5 mL eppendoff and was calculated freezing point depression osmometer.The osmolarity of pharmaceutical preparation was turned out to be 272 mOsmol/kg.

6. In-vitro permeation studies :

Franz diffusion apparatus (Orchid Scientific & Innovative India Pvt. Limited, Nashik,Maharashtra) was put into application to to investigate the *in vitro* release of radicava in nano suspension and prepared in-situ emulsion gel through activated dialysis membrane into synthetic eye micro-environment . The drug release testing was performed in pH 6.4 with dissolution media which is simulated tear fluid (STF) (Table 3) at 37°C (corneal temperature). The tear fluid was prepared one – night prior before experiment and was preserved at 4°C . Cellulose acetate semi-permeable membranes (Sigma- Aldrich Dialysis Membrane 12 Kilodalton) were used as the artificial membrane. (Figure6.1)

Components	**Weight(gram)/Volume**
Sodium chloride	0.670
Sodium bicarbonate	0.2
Calcium chloride dihydrate	0.008
Purified water	**100 mL**

Table 3: Simulated Tear Fluid Composition

Activation of dialysis membrane :

The dialysis membrane was washed under continuous tap water for 4 hours. In order to remove sulphate salts, the membrane was dipped entirely into 0.3% (w/v) sodium sulphide solution at 80 °C for a minute. It was then washed with hot water (60°C) for 2 minutes followed by membrane acidification with 0.2% (v/v) sulphuric acid. The activated membrane must be well preserved in distilled water at 4°C.

Permeation Experiment :

Vertical Franz diffusion cells with a volume of 35 mL (contact area/diffusion area: 3.14cm^2) were used. Cellulose acetate film- like membranes were fixed carefully at diffusion area of receptor chambers to restrict occurrence of air bubbles of the cells following the addition of the release media. Therefore, dissolution media must be completely brimmed in receptor chamber and must be in contact with dialysis membrane.0.5% tween 80 was added release media to imitate SFTA3 surfactant protein in an eye. Afterwards, donor chambers were mounted on the membranes and clamped tightly. 109.3 mg (weight/volume) which is equivalent to two drops of in-situ gel and

1000 µL of nanoemulsion were loaded onto synthetic membrane. The stirring speed of the Franz diffusion cells was set at 550 rpm. At pre-determined time intervals (15 minutes,45 minutes, 1 hour, 2 hours, 3 hours, 4 hours, 5 hours, 6 hours, 7 hours and 8 hours) 1 mL of the media were withdrawn from the receptor chambers and replenished with fresh media.

7. Ex -vivo permeation studies :

Caprine Corneal Preparation :

Freshly excised whole eyeballs of goat were procured from local butcher's shop to laboratory in cold (4°C) in phosphate buffer saline within 1 h of slaughtering. The goat eye corneas were dissected with care along with 2–4 mm of surrounding sclera tissue from the eyeball. The dissected ophthalamic tissue was washed with cold saline in order to remove adhering pigments as shown in Figure 5. The corneal tissues were preserved in cold phosphate saline buffer (Table 4) with pH 6.4 which fabricates sterile medium.

Components	**Weight(gram)/volume (300mL)**
NaCl (137 mM)	1.75
Na_2HPO_4 (8 mM)	0.44
KH_2PO_4 (2Mm)	01.75
Millipore water(Dispersant)	**300 mL**

Table 4 : Composition of phosphate buffer saline

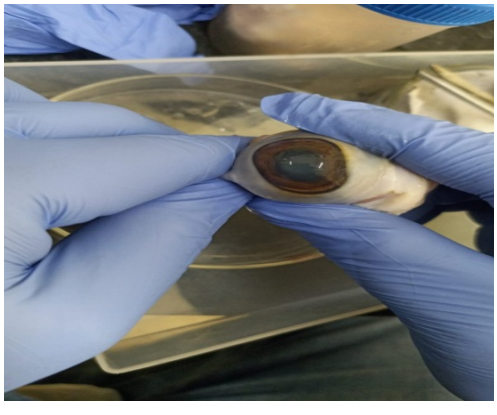


Figure 13 : Caprine ex-vivo model

Permeation Experiment

Freshly slaughtered corneas obtained by the above procedure were mounted on the modified Franz diffusion apparatus by sandwiching the scleral tissues between the clamped donor and the receiver chamber. Convex lens shape must come in contact with sample formulation. The receiver fluid was maintained at 37°C with the help of warm water circulation and kept under stirring using a Teflon coated franz diffusion rice magnetic bead. The concentration of permeated drug at the defined time intervals (15 minutes,45 minutes, 1 hour,2 hours,3 hours,4 hours) was determined plotting standard curve.(Figure7.1)

Permeation % = Amount of drug permeated in receptor/Initial amount of drug in donor * 100

8. Confocal Microscopy :

Preparation of blank formulation :

The sample formulations nanoemulsion and in-situ emulsion gel were made without drug with addition of 50 ppm Rhodamine B dye concerntration.

Permeation Experiment :

The Rhodamine B loaded blank sample formulations were loaded onto scleral tissue of goat eye sandwiched between donor and receptor compartments of franz diffusion assembly.The permeation was performed for 4 hours . Afterwards, permeated scleral tissues were preserved for confocal microscopic treatment in 10% formaldehyde solution.(Figure 13)

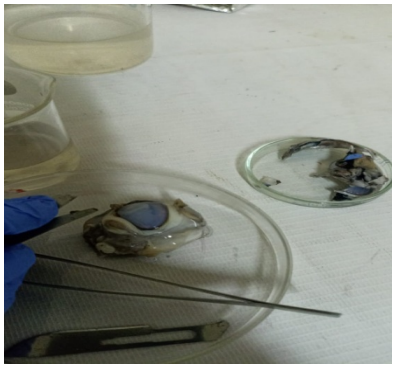


Figure 13 : Sample Preparation for confocal microscopy

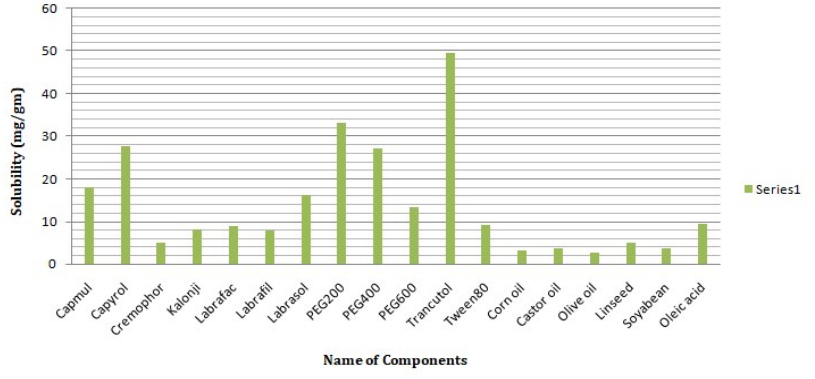
Sample preparation for confocal microscopic analysis :

The thin sections of scleral tissues of permeated goat eye were cut and placed onto clean glass slide for cryotomy. The sections were only using cryostat (Leica Biosystems) when it was equipped with -20°C temperature. The tissues were fixed in glass slide in cryotome using tissue fixing gum and sections were cut at thickness of 5 μm were

visualized under confocal microscope (LEICA CM 1950). Live results were extracted with the help of LASX software.(Figure 17 : Confocal microscopy (a) In – situ emulsion gel (b) Nanoemulsion)

9. Results :

The kalonji oil was taken among all other oils based upon it's therapeutic profile with negligible toxicity when administered in a prescribed format along with its considerable drug solubility .(Graph 1)



Graph 1 : Solubility Experimentation (Biostatistics)

The protein stabilized nanoemulsion and in –situ emulsion gel was evaluated for physical characterstics and it was observed in light – yellow colour with characterstic aroma of black cumin oil. It exhibits no precipitation, turbidity and phase separation. Emulsion was found to be uniform all – over and in – situ gel turned out to be consistent.

The 40 % oil concerntration which is (400 µL) was optimized because of its particle globule size of 252.2 nm and polydispersity index of 0.26 (Figure14).With the combination of 40%oil concerntration 1% protein solution concerntration was finalized with particle globule size of 252.5 nm and polydispersity index of 0.26 (Figure15).
The pH of protein stabilized nanoemulsion and in situ emulsion gel was determined using a pH meter was observed to be 6.44 at room temperature. The rheological studies were performed using viscometer and pseudo-plastic nature in- situ gel was confirmed. (Figure16). The spreadibility of emulsion gel formulation was found to be 1090.598.

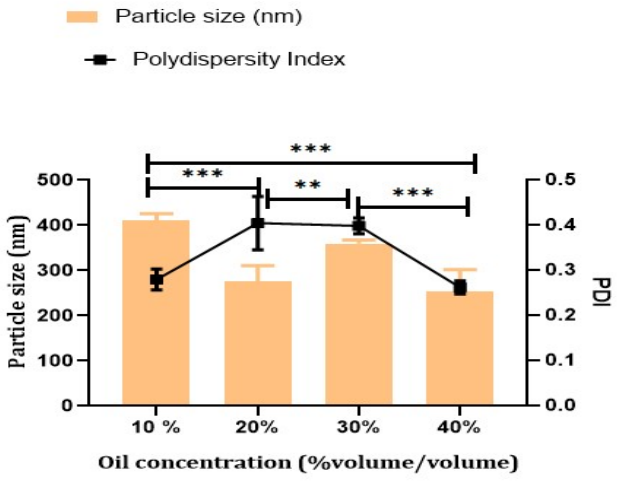


Figure 14: Particle size and polydispersity index of protein solution concerntration

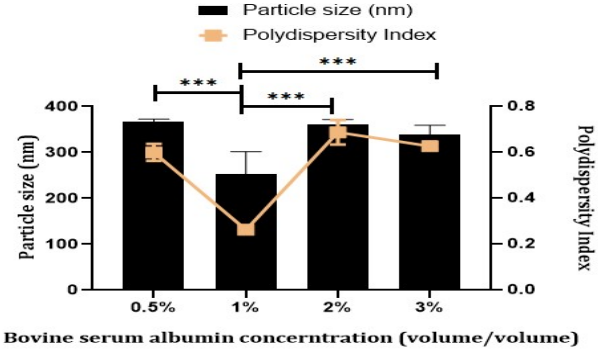


Figure 15 : Particle size and polydispersity index of oil concerntration

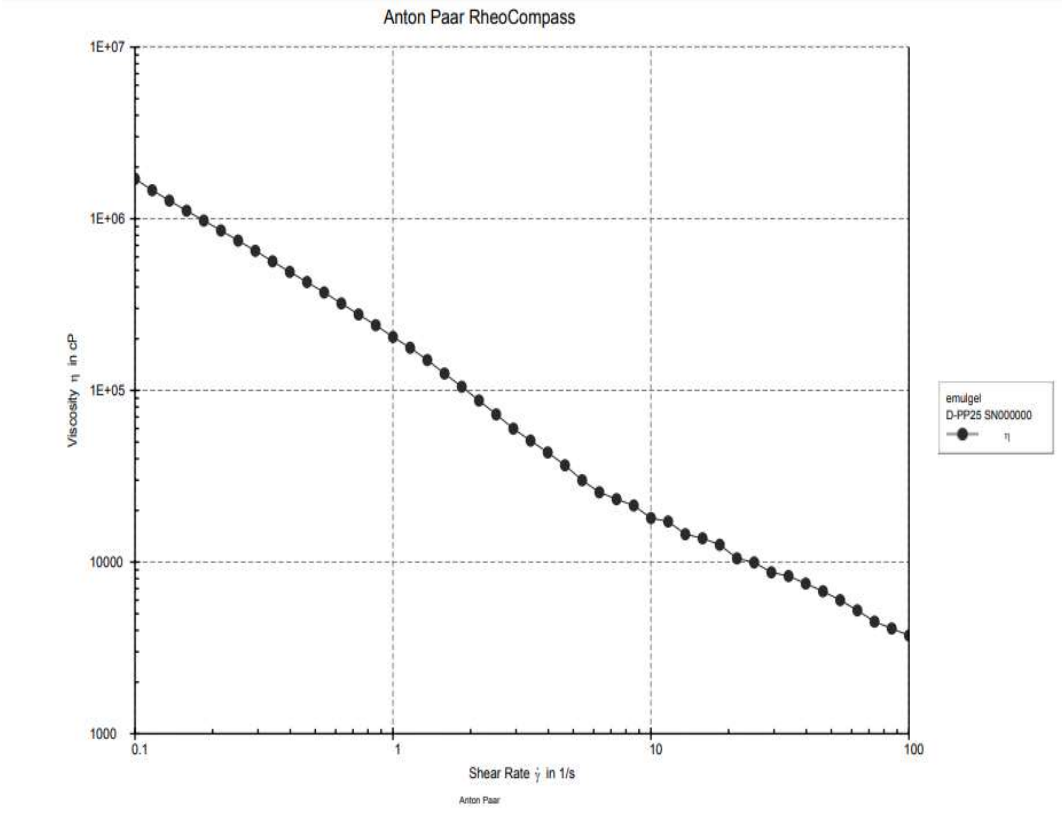
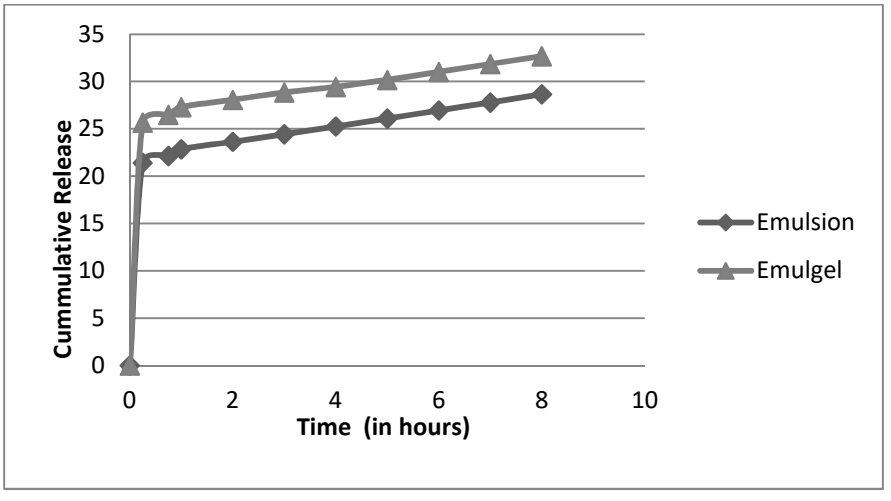
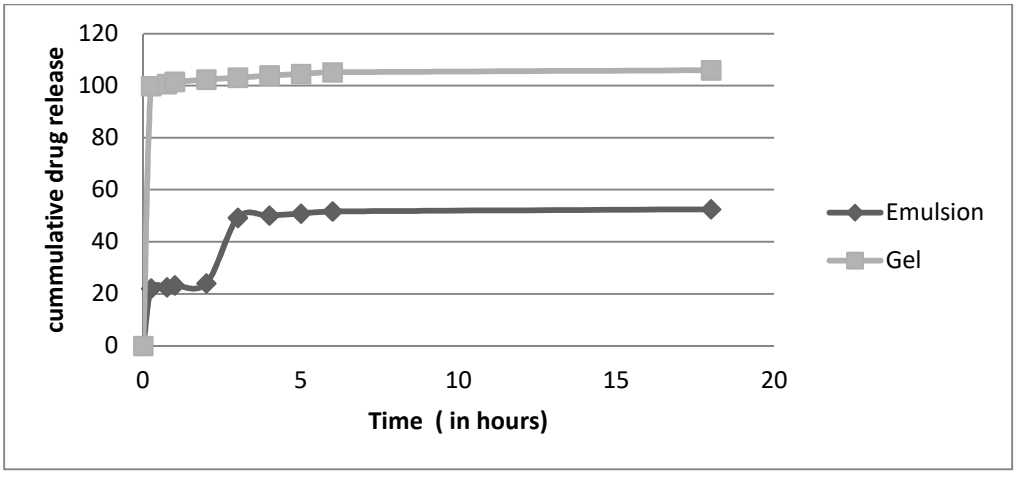


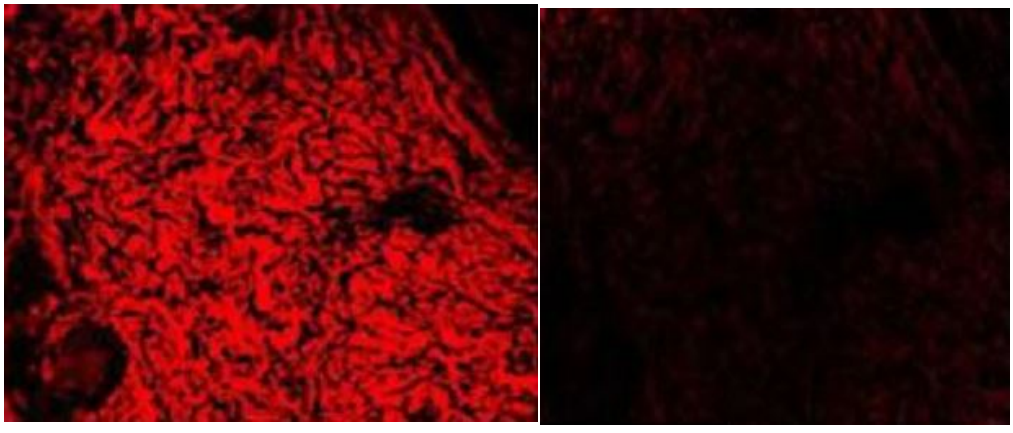
Figure 16 : Rheological studies



Graph 2 : In– vitro permeation experiment



Graph 3 : Ex- vivo permeation



(a) **(b)**

Figure 17 : Confocal microscopy (a) In – situ emulsion gel (b) Nanoemulsion

RESULTS

Test ID	Batch	Firmness g Firmness	Work of shear g.sec (Traditional) F-T
Start Batch gel	gel		
gel2	gel	1090.598	636.684
End Batch gel	gel		
Average:	gel (F)	1090.598	636.684
S.D.	gel (F)		
Coef. of Variation	gel (F)		
End of Test Data			

Thursday, 13 July, 2023
Page 2 of 2

Figure 18 : Texture analysis

Conclusion :

Improvements in the efficacy of ophthalmic delivery systems have resulted from a better comprehension of the principles and processes governing ocular drug absorption and elimination, as well as ongoing technological advances. Compared to conventional ocular dosage forms, the use of in situ gel laden with polymeric nanoparticles offers a number of benefits. Sustain and protracted release make the delivery system more trustworthy. Utilising biodegradable and water-soluble polymers in nanoparticle-loaded in situ gel formulations improves their acceptability and efficacy as drug delivery systems. In situ activated gel-forming systems are favoured because they can be administered in the form of a drop and cause significantly less vision-related discomfort.