Development and characterization of mixed gel based hydrogel, emulgel and bigel.

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Under the supervision

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CERTIFICATE

This is to certify that the thesis entitled, **"Development and characterization of mixed gel based hydrogel, emulgel and bigel"** submitted by **Mr. Senggam Wakhet Singpho** in partial fulfillment of the requirements for the award of Master of Technology in Biotechnology and medical Engineering with "Biotechnology" specialization during session 2012-2014 in the Department of Biotechnology and Medical Engineering, National Institute of Technology, Rourkela.

It is an authentic work carried out by him under my supervision and guidance. To the best of my knowledge, the matter embodied in this thesis has not been submitted to any other University/Institute for the award of any Degree or Diploma.

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DATE: **Senggam Wakhet Singpho**

Abstract

The present study was based on development of gel formulations of agar-gelatin hydrogels, emulgels and bigels which were evaluated as delivery modules for metronidazole (MTZ). Agar and gelatin were used for the preparation of mixed food hydrogels. Soyabean oil (SO) was used for the preparation of emulgels and was incorporated within the prepared hydrogel. Organogel was prepared using soyabean oil as a solvent and stearic acid (SA) as organogelator. The bigel was prepared by mixing organogels with the agar-gelatin hydrogel. The prepared gels were characterized for their surface morphology, mechanical properties, chemical interactions and electrical properties. Field emission scanning electron microscopy was used to visualize the surface topography. XRD studies established the amorphous nature of the gels. FTIR study confirmed the interpolymeric bonding between gelatin and agar as well as encapsulation of soyabean oil within the polymeric matrix. There was no interaction between the polymers and drug. The hydrogels showed lower impedance than emulgels and bigels indicating higher content of aqueous phase within it. The hemocompatibility test confirmed the blood compatibility of the preparations. The emulgel showed least mucoadhesive characteristic while the hydrogel sample showed highest mucoadhesion. The leaching study confirmed the presence of oils in the samples. The swelling profiles for the formulations were in tune with i*n vitro* release studies at pH 7.2 phosphate buffer. Metronidazole was used as a model antimicrobial drug to incorporate within the preparations. The antimicrobial efficiency of all the drug loaded formulations was found to be equivalent.

Keywords: Hydrogel, hemocompatibility, emulgel, bigel, mucoadhesion

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NOMENCLATURE

Description	Symbol or short form
X-Ray Diffraction	XRD
Fourier Transform Infrared Spectroscopy	FTIR
Differential Scanning Calorimetry	DSC
G1	Hydrogel
G2	Emulgel
G3	Bigel
MTZ	metronidazole

Chapter 1

Introduction

1. Introduction

The behavior of composite food materials are of special interest and sometimes look curious from the common knowledge obtained from non-food composite materials. The resultant mixture of most synthetic polymers has properties that lies intermediate of its elementary constituents. However it is not the case for food gel combinations as their combination is vastly dependent on the aqueous content of the mixture which dilutes the polymeric constituents in the mixture and may vary with different compositions. The present study used two biopolymers for the preparation and characterization of hydrogels, emulgels and bigels.[1] Hydrogels are highly absorbent 3-dimensional crosslinked polymeric networked structure capable of mimicking living tissues in terms of elasticity and mechanical properties. They are insoluble in water due to presence of physical and chemical crosslinks which also provides the networked structure and physical integrity. [2]The polymers used are hydrophilic and maybe of both synthetic and natural in origin.[3]Synthetic polymers required can be adjusted as per compositional requirement as well as properties like degradation rate, mechanical and chemical properties. Natural polymers provides networked structures resembling living tissues and has lower biocompatibility issues. Agar is a hydrophilic polysaccharide and chemically made up of alternating 3-O-linked D – galactopyranose and 4-O-linked 3,6-anhydro-L-galactopyranose.[4] Gelatin is a natural polymer containing many glycine, proline and 4-hydroxyproline residues. These biopolymers are biodegradable and finds applications in areas of cosmetics, food industry, pharmaceutical and medicine.[4] Emulgels are biphasic systems either of oil-in-water or water-in-oil type. Usually the oil phase is added along with surfactant onto the gel phase. The resultant emulgels is a result of emulsion formation between the oil phase and water phase in the gel base under the influence of surfactant under stirring conditions.[5]The oil-in-water emulsion is suitable for incorporating

hydrophobic drugs whereas water-in-oil type emulgels find wide applications in treatment of dry skin and moisturizing applications.[6] Soyabean oil (SO) is used as the oil phase for preparation of emulgels. The drug is incorporated into the oil phase and then onto the aqueous gel base.[7] Bigels are formulations prepared by mixing organogel (lipophilic phase) with hydrogels (aqueous phase) and are stabilized using various surfactants.[8] Soybean oil is one of the most abundant vegetable oil with a high composition of linolenic acid extracted from soyabean seeds. This oil has wide applications in food industry as an edible oil whereas it has also been recognized in the pharmaceutical industry as an active ingredient.

The present study is focused on the study of agar-gelatin composite gels as an ideal controlled drug delivery vehicle and its mechanical properties. Soyabean oil (SO) was used for the development of the emulgels and bigels. Stearic acid and soyabean oil was used for preparation of organogel to be encapsulated within agar and gelatin. Metronidazole was used as model antimicrobial drug. The efficacy of the antimicrobial effect of the drug loaded formulations were tested against *E. coli*.

Chapter 2
Literature Survey & Objectives

Gel Systems:

The study of interaction between materials of different physical state and chemical nature provide us with good insight of the property of the individual materials and their viability towards development of a desirable product or system. A gel is a solid system which can immobilize or encapsulate a solvent within its structure[2]. A gelling system is characterized by 3-dimensional matrix and holds the solvent component within this structure. The solid system is polymeric and maybe natural or synthetic in nature. The dispersed solvent can be of either hydrophilic or lipophilic in nature. Based on the liquid component entrapped the gel system is of two types:

a) Hydrogels: Hydrogels are made up of water soluble homopolymers or copolymers and possess three dimensional crosslinked networked structures. The hydrogel maintains its structural and physical integrity inspite of holding water due to the chemical and physical crosslink interactions that results in either covalent bonded or hydrogen bonded structures respectively[9-10]. The presence of hydrophilic functional groups like O-H, N-H, COO, etc in its polymer chain allows the hydrogels to hold large amount of water within it[11]. The water content also makes it soft and rubbery making it vulnerable at the same time strong enough to withstand stress similar to our body tissues [3]. Hydrogels are porous in nature and finds applications in wound healing as well as carriers in drug delivery modules for swelling-controlled drug release[12].

b) Organogels. Organogels are three dimensional crosslinked networked structures that immobilize organic solvents within its gelator matrix [13]. The matrix may be fluid filled matrix or a solid matrix. The interaction between the polymer base and lipid base is stabilized by

substances which acts to reduce surface tension and allows their interaction (span 60, tween 80). These are semi-solid systems and possess solid like rheological properties at room temperature.

Emulgels

Emulgels may be defined as biphasic systems comprising an apolar internal phase (emulsion) within an aqueous gel base. The emulgel system is a novel approach for drug delivery applications especially for hydrophobic drugs. The hydrophobic drugs is mixed in the oil phase which is later incorporated within the conventionally stable gel base [7].

Bigels

Bigels are another class of biphasic crosslinked systems that is made up of the oleogels/organogels as the internal phase within the continuous aqueous phase of hydrogels [8]. Bigels were formulated as a novel approach for developing modules for topical drug delivery applications and their increased bioadhesion properties plays a big role in that [14].

Agar and gelatin were used as ideal biopolymers for the above mentioned semi-solid systems. Agar is a hydrophilic colloid consisting of polysaccharides that have the ability to form reversible gels simply by cooling a hot aqueous solution. It is composed of alternating 1,3-linked d-galactose and 1,4-linked 3,6 anhydro-l-galactose units [15]. Gelatin,on the other hand, is derivated from collagen,which has shown wide- spread applications in biomedical fields primarily due to its excellent biocompatibility and low immunogenicity [16].

Analytical Methods

Different analytical methods were employed to characterize the properties of the above mentioned gel formulations. The formulations were characterized for:

a) Physicochemical properties

   The physicochemical properties give hindsight into the physical and chemical properties of the formulations. The physical properties include studying the thermal profile using differential scanning calorimeter (DSC). The amorphous and crystalline nature of the gel system is studied using x-ray diffraction (XRD) techniques. The chemical interactions are analyzed using Fourier Transform Infra-Red (FTIR) Spectroscopy.

b) Mechanical Properties

   The gel systems are semi-solid in nature and therefore possess viscoelastic properties. Stress relaxation method is used to study the viscoelastic nature. Here the sample is compressed by applying a certain amount of force. The stress developed is applied for a fixed duration after which the force is removed.

c) Electrical properties

   The electrical properties investigate the conductivity of the sample in a non-destructive manner. The study also indicates the amorphous nature of the samples.

d) Biological Activity

   The biological activity studies the mucoadhesion, biocompatibility, drug release and antimicrobial properties. Suitable drugs are incorporated within the formulations to study the drug carrying ability *in vitro*.

Objectives:

1. To develop and carry out an in-depth characterization of agar-gelatin based phase separated hydrogels, emulgels and bigels.

2. To thoroughly characterize the above formulations by physicochemical, mechanical, electrical and biological activity studies.

Chapter 3
Materials and Methods

3. Materials and Methods

3.1. Materials:

Agar-Agar powder (Grade 400 GS) was purchased from Marine Chemicals, Cochin India. Gelatin powder, Stearic acid, Tween 80 and Glutaraldehyde (25%) were purchased from Loba Chemie, Mumbai, India. Refined soyabean oil (Gokul ®, Gujarat, India) was purchased from the local market. Metronidazole was procured from Aarthi Drugs (P) Ltd, Mumbai, India. Fresh goat blood and small intestine were procured from local butcher shop. Double distilled water was used throughout the study. All the experiments were done in triplicates.

3.2. Preparation of Gels:

Preparation of Hydrogels (G1)

2%(w/v) agar powder was suspended in distilled water and autoclaved at 15 atm and 121℃. 20%(w/v) gelatin solution was prepared by suspending the gelatin powder in distilled water which swelled at 65℃ in a water bath.[1] The suspension was further stirred at 300 rpm at 65℃ to get final gelatin solution. Both the agar and gelatin solution were taken in 1:1 ratio and mixed at 65℃ and 300 rpm. The mixture so formed was cross linked with glutaraldehyde reagent (0.5 ml) and immediately allowed to cool off. The cross linking resulted in the solidification of the gelatin and agar layer thereby resulting in the formation of hydrogel.

Emulgels (G2)

The molten agar and gelatin gels were mixed in 1:1 ratio as earlier and stirred at 65 ℃ and 300 rpm. 0.5 ml of Tween-80 is added as surfactant followed by addition of .5g of soybean oil under stirring conditions. Glutaraldehyde reagent was used to cross link the emulsion formed which

was immediately poured into petriplate or cylindrical moulds and allowed to set. Drug loaded emulgels were prepared in a similar manner. The drug loaded emulgels contained metronidazole in the concentration of 1% (w/v) of the internal phase.

Organogel (OG)

Organogels was prepared by dissolving stearic acid (19% w/w) in soybean oil (81% w/w) by vortexing in culture vials. The vials were then kept in a water bath at 60℃ until a homogeneous clear solution is formed. The hot solutions were cooled at room temperature to allow organogel formation.

Metronidazole (1% w/w) was dissolved along with stearic acid to formulate medicated organogel preparations.

Bigels (G3)

The two biopolymers were taken in earlier compositions and conditions as in emulgel preparation. 2.5 g of the organogels was incorporated within the mixture to get both drug loaded and unloaded bigel preparations.

Table 1. Composition of gels (in grams)

Sample	Agar	Gelatin	Soyabean oil	Organogel	Metronidazole
G1	10	10	-	-	-
G2	8.75	8.75	2.5	-	-
G3	8.75	8.75	-	2.5	-
G1D	10	10	-	-	0.2

G2D	8.75	8.75	2.5	-	0.2
G3D	8.75	8.75	-	2.5	0.2

3.3. Microscopy studies

The gel microstructures were visualized under bright field microscope (LEICA-DM 750 equipped with ICC 50-HD camera, Germany). The samples were prepared by mounting a drop of the molten uncross-linked solutions of all three compositions (G1, G2, G3).in glass slides and enclosed with a cover slip.

The surface morphology of the gels was studied under field emission electron microscope (FEI, Nova NanoSem 450). The samples were prepared by drying the gels for 48 hours at 45℃. The surface was observed under 500X magnification at 10 kV.

3.4. X-Ray Diffraction studies

The XRD analysis was done using X-ray diffractometer (XRD-PW 1700, Philips, Rockville, USA). The samples were sized into pieces of 1 cm x 1cm and dried for 48 h prior to analysis. The gel samples were scanned from 5º-50º at 2º per min with Cu Kα radiation as the source. The analysis was done inorder to study the crystallinity of the gel samples.

3.5. Leaching Studies

The leaching of the internal phase was qualitatively studied using filter paper method.[17]In short, circular slabs of 1.9 cm diameter of all three formulations (G1, G2, G3) was kept on a filter paper and incubated at 37℃ for 24 h. The quantification of the leaching of the internal phase was done as per the reported literature. [18] Accurately weighed 100 mg of the gels (W1) was soaked in 1.0 ml of water (W2) for 30 min at room temperature in a 2 ml centrifuge tube. The tubes were centrifuged at 10000 rpm for 2 min (Tarsons, MC-02 Spinwin). The supernatant was collected and subsequently dried in a hot air oven at 55oC for 48 h. The weight of the dried supernatant was measured accurately (W3).[18]

The percentage of leaching was calculated as:

$$Percentage\ of\ \ leaching = \frac{W_3}{W_1} \times 100 \qquad (1)$$

3.6. Hemocompatibility

The hydrogels prepared with biopolymers are generally biocompatible.[19] The biocompatibility of the prepared agar-gelatin gels was analyzed by determining the % hemolysis of the goat blood in the presence of the gel samples. The sample for the test was prepared by adding 0.5 ml of diluted blood followed by addition of 9 ml of phosphate buffer saline in falcon tubes. The gel samples were cut into dimensions of 1cm x 1cm and placed in these tubes. Positive and negative control was prepared in the same manner by taking 0.5 ml of 0.01 N HCl and 0.5 ml of phosphate buffer saline, respectively. The centrifuge tubes were then incubated at 37ºC for 60 min. The samples and the controls were centrifuged at 3000 rpm for 10 min. The O.D. of the

supernatants was taken at 545 nm using a UV-Visible spectrophotometer. The % hemolysis is calculated as per the following formula [20].

$$\% \, Hemolysis = \frac{OD_{test} - OD_{negative}}{OD_{positive} - OD_{negative}} \times 100 \, \% \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad (3)$$

where,

ODtest= optical density for the test sample

ODpositive= optical density for the positive control

ODnegative= optical density for the negative control

3.7. Mucoadhesion Studies

The mucoadhesive property of the agar-gelatin gel samples was analyzed as per the modified tablet disintegration method [21] and mechanical tester. The mucous membrane of goat intestine was used for study. The mucosal membrane was laid exposed on a glass slide such that the gel samples (1cm x 1cm) were placed on it. Thereafter the slides were placed on USP disintegration baskets. Phosphate buffer saline (pH=7.2) was used as disintegration medium. The study was conducted for 24 hours and time required for the detachment of the gels from the mucosal surface was noted down.

The texture analyzer (Stable Microsystems, TA-HD plus, U.K) was used to study the mucoadhesive strength of the gel samples. The gel samples (5mm x 5 mm) were attached to the surface of the cylindrical probe (30 mm diameter) with double sided acrylate tape. The goat intestine was attached onto the aluminium platform of the texture analyzer such that the mucosal surface was exposed and set to meet the probe with the gel sample.

3.8. *In-vitro* drug release

The *in-vitro* drug release was done using a vertical diffusion cell arrangement to analyze the release profile of metronidazole from the polymer matrices. The gel samples were cut in 5 cm x 5 cm and placed in donor compartment separated from the receptor by a dialysis membrane (molecular weight cut off= 60 kDA) maintained at 37℃ under stirring conditions of 100 rpm in a magnetic stirrer (Remi India Ltd, Mumbai, India). Phosphate buffer (pH=7.2) was used as dissolution media and was replaced with fresh media after every interval of 30 min. The study was conducted for 10 h. The dissolution media was analyzed by UV-visible spectrophotometer (UV-3200, LabIndia , India) at 321 nm.

3.9. Antimicrobial Assay

The antimicrobial efficiency of the metronidazole loaded agar-gelatin gels was tested against *E. coli* (NCIM-5051) by disc diffusion technique. Circular blank and drug loaded gel discs of 9.0 mm diameter were placed on the nutrient agar surface on the culture plates. The culture plates were incubated at 37 ºC for 12 h.

Chapter 4
Results and Discussion

4. Results and Discussion.

4.1. Preparation of gels

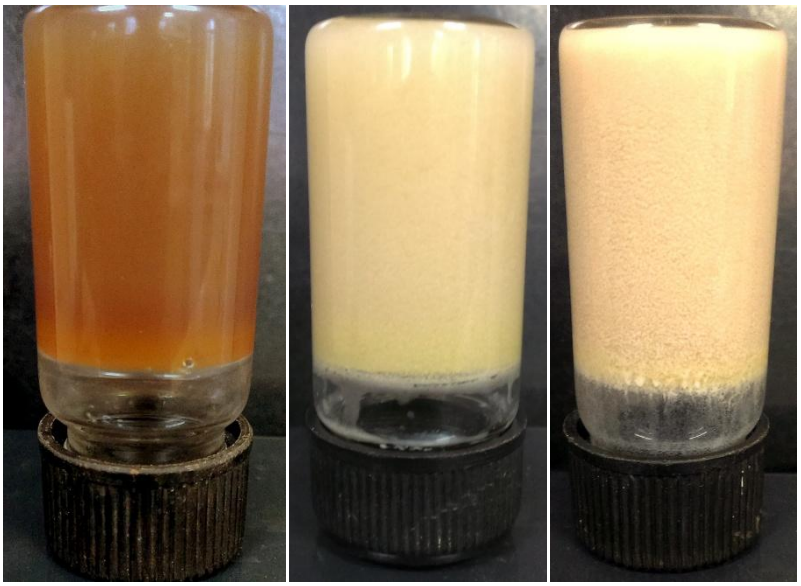

Figure 1 showing pictographs of G1, G2, G3.

The G1 sample containing agar and gelatin was homogeneous brownish red .G2 and G3 were opaque and exhibited light brownish yellow. The G2 surface was smooth while G3 appeared granular.

4.2. Microscopy Studies

The micrographs of the molten gel samples have been shown in Figure 2. G1 exhibited a smooth surface texture indicating homogeneity of the biopolymeric mixture. G2 micrographs suggested the presence of dispersed circular soyabean oil droplets in the within the agar-gelatin continuum gel base. The oil droplets in the bigel micrograph (G3) were aggregated and were present as clusters throughout.

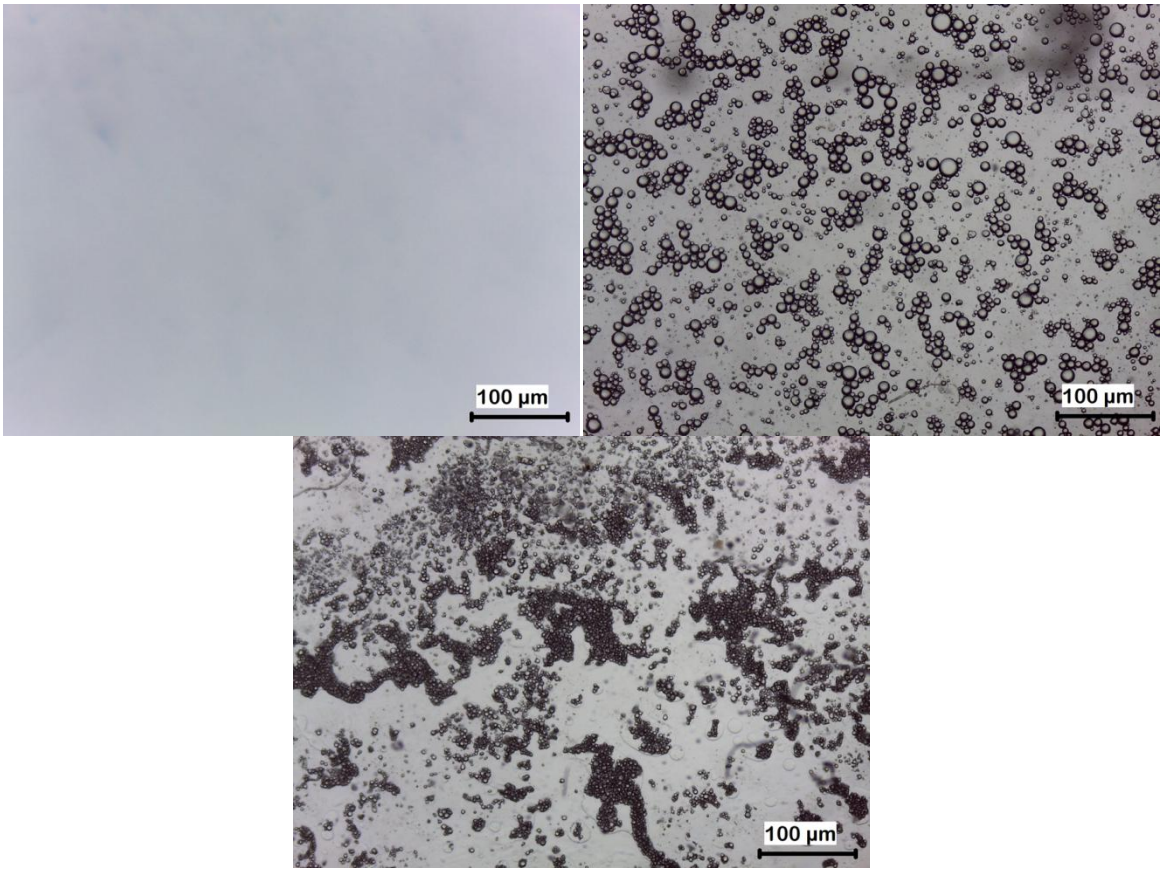

Figure 2 showing micrographs of G1, G2 and G3

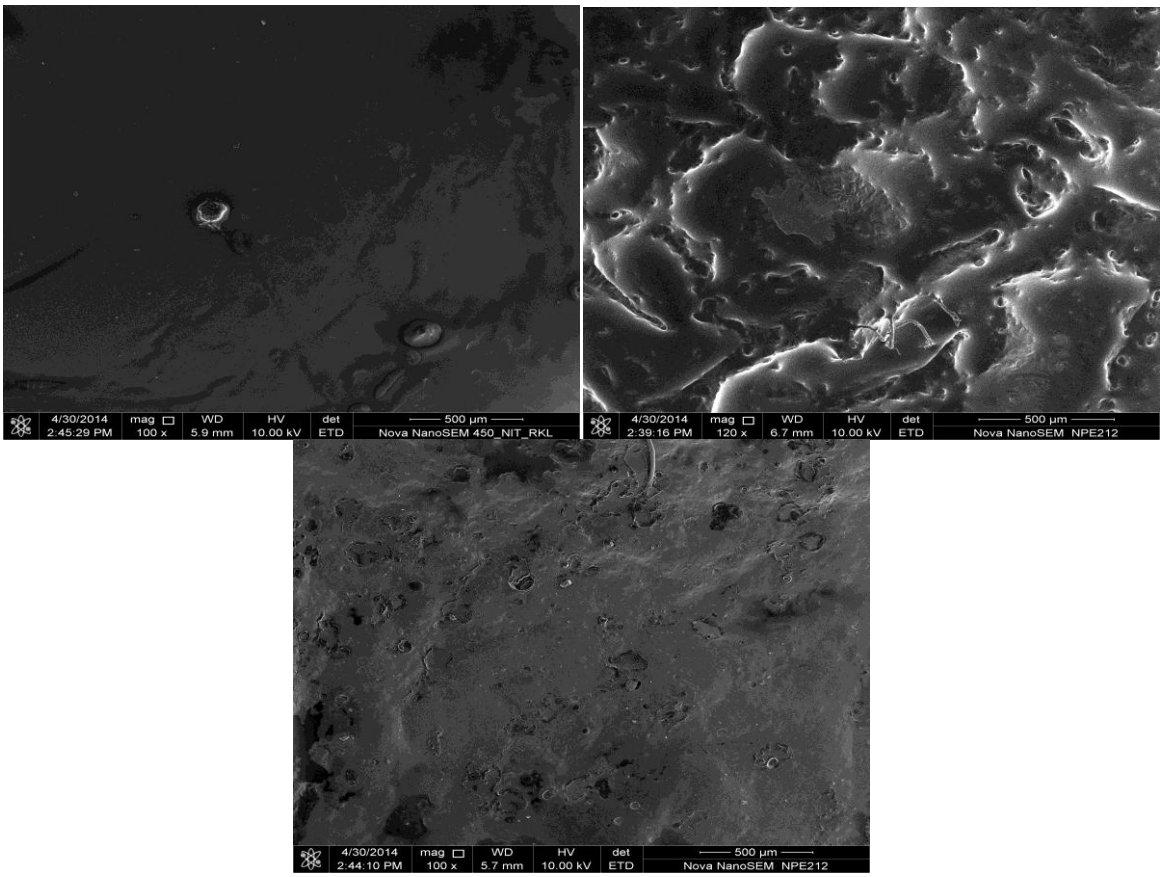

Figure 3 showing FESEM images of G1, G2 and G3.

The surface morphology of the samples were examined under field emmision scanning electron microscopy (FEI, Nova NanoSEM 450) at 500X and 10 kV. G1 exhibited smooth surface indicating complete co-polymerization. G2 and G3 exhibited uneven surface indicating dispersal of the apolar phase. G3 also demonstrated spherical and spindle shaped structures on the surface.

4.3. XRD

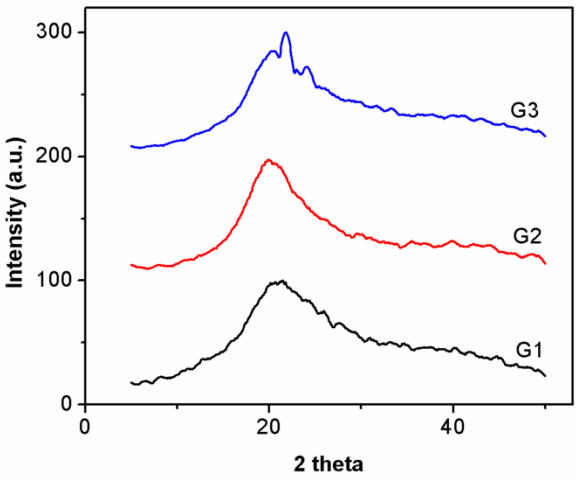

Figure 4.XRD diffractograms of gels.

The normalized x-ray diffractograms for the agar-gelatin gels showed a broad peak at ~ 20º 2θ. The XRD pattern indicated that there was an increase in the crystallinity in the bigel as compared to emulgels and bigels. The FWHM values were in order of G1>G2>G3. In general, higher the FWHM and AUC, higher is the amorphous nature of the gels [22]

4.4. Leaching studies

The leaching of the internal phase from the formulations was qualitatively studied by filter paper (Figure). The test is based on the principle of absorption of oil (apolar phase) by the filter paper, which results in the formation of a dark zone where the oil is absorbed. No dark zone was observed in G1 as there was no internal phase. The diameter of the dark zone was higher in G2

as compared to G3. This suggested that the leaching was higher in G2 as compared to G3. The quantitative leaching study also confirmed the results obtained by the qualitative studies. The leaching was 20% in G2 and 10% in G3.

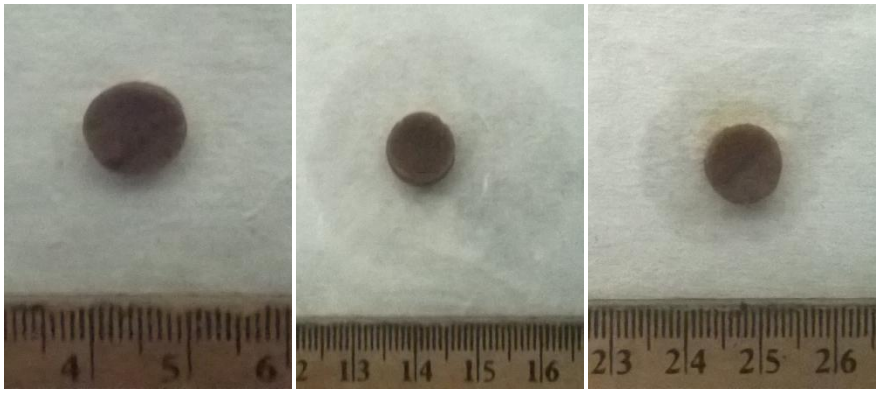

Figure 5: Showing qualitative leaching of G1, G2 and G3 respectively.

Table 2 showing % leaching for quantitative study

Sample	% Leaching
G1	-
G2	20
G3	10

4.5. Hemocompatibility Studies

The hemocompatibility study was done to study the hemolysis of the blood cells in the presence of the formulations. Presence of hemolytic components releases hemoglobin into the continuous phase, which results in the yellowish coloration of the continuous phase. The amount of hemoglobin released was then analyzed spectrometrically. The study showed that the %

hemolysis for all the three formulations were <5%. This suggested that the formulations were highly hemocompatible in nature.

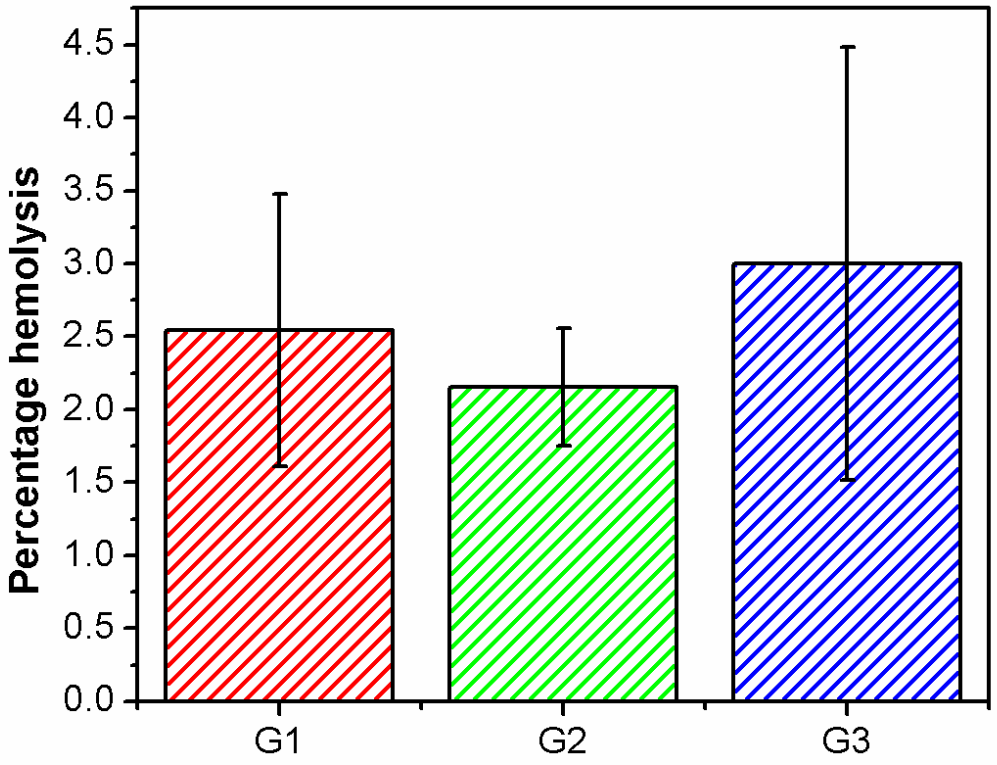

Figure 6 : Showing %hemolysis in the presence of the gel formulations.

Table 3 :showing % hemolysis values

Samples	% Hemolysis
G1	2.54±0.93
G2	2.15±0.40
G3	3.0±1.48

4.6. Mucoadhesion Studies.

The results of the in-vitro wash off method suggested that the mucoadhesion time for the formulations was ~20 h. This indicated that the incorporation of the internal oil/organogel phase did not significantly alter the mucoadhesive property of the formulations. The differences in the mucoadhesion timings were statistically insignificant ($p > 0.05$). Though the mucoadhesive timings did not vary insignificantly, the work done (from the mechanical tester) to detach the formulations was found to be highest in G1 followed by G3 and G2, respectively. The differences in the work done were not significant for G2 and G3. This can be attributed to the leaching of the internal phase from G2 and G3 as was evident from the leaching studies.

Table 4 : showing adhesion time (h)

Samples	Time (h)	AUC
G1	21.91±132	7.50±2.76
G2	19.21±257	5.63±0.48
G3	20.18±216	4.96±1.81

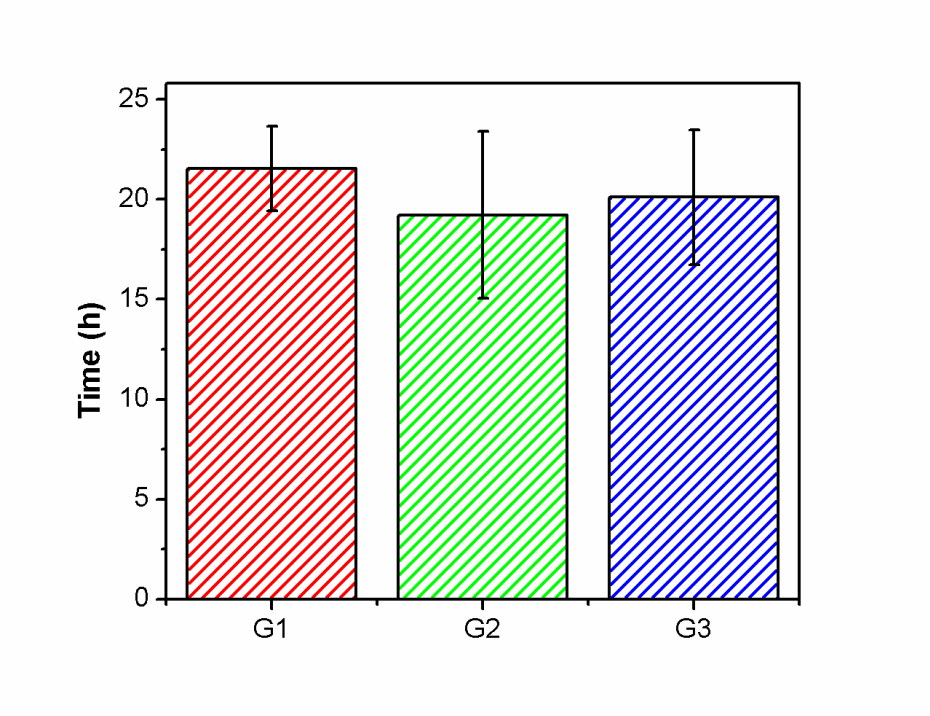

Figure7: Showing detachment time for the gel formulations by wash off method.

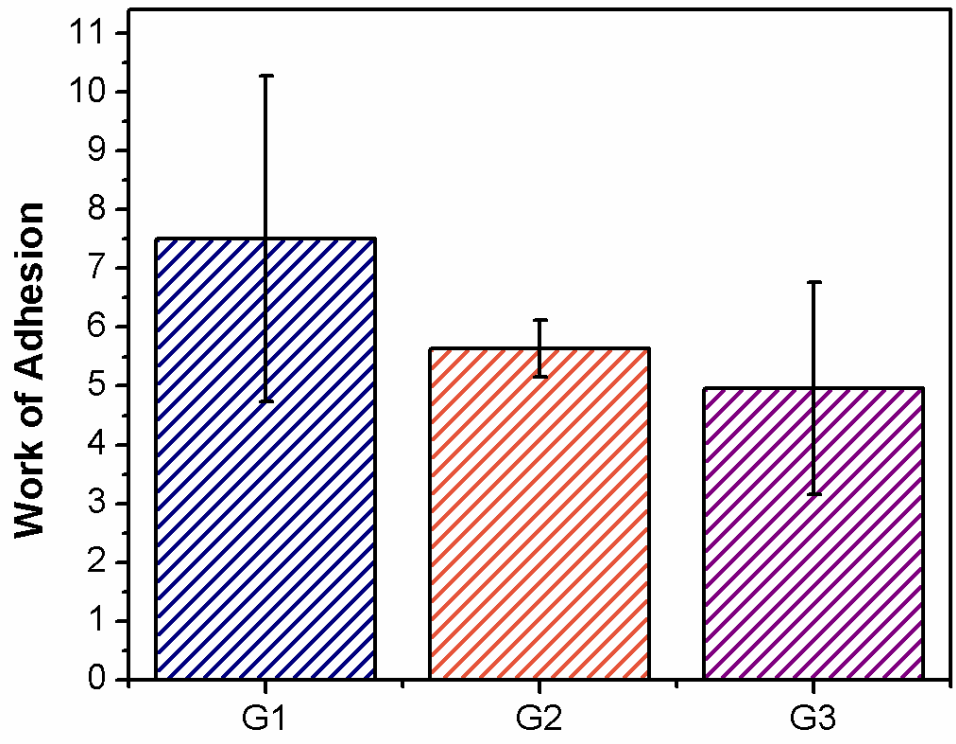

Figure 8: showing work of adhesion for the gel formulations.

The area under curve is function of work done to detach the gel sample from the mucosa. From the table we can conclude that the order of mucoadhesion is G1>G3>G2 and is consistent with the results obtained from the wash-off method.

4.7. *In-vitro* drug release

The *in-vitro* drug release profiles of the samples are given below. The cumulative percentage drug release (CPDR) of G1D, G2D and G3D was ~45.43%, ~44.82% and ~42.28% respectively. The release was in accordance to the swelling study in the same pH (7.2 phosphate buffer). The correlation coefficient (r^2) was employed to study the best fit model. The diffusion of the drug followed non-fickian kinetics for all three formulations.

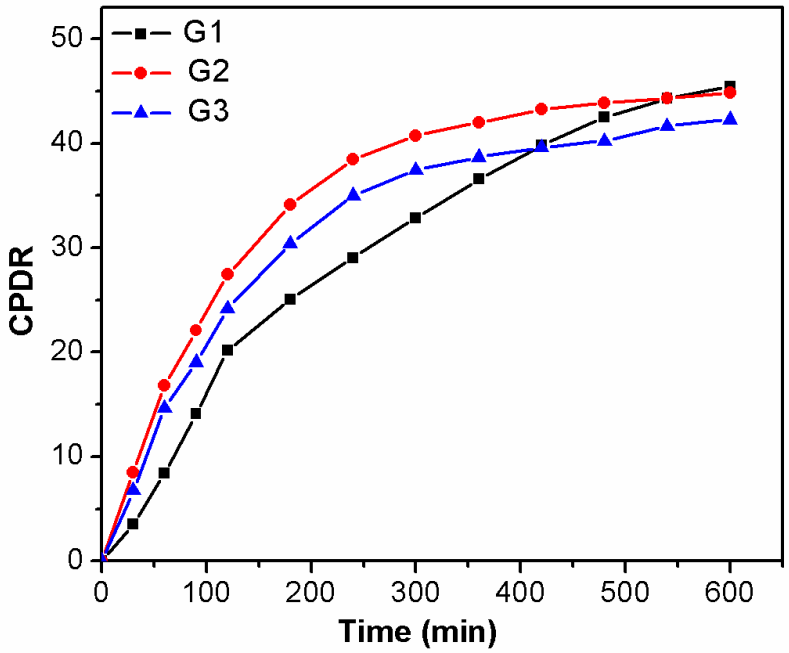

Figure 9: Drug release profile of formulations

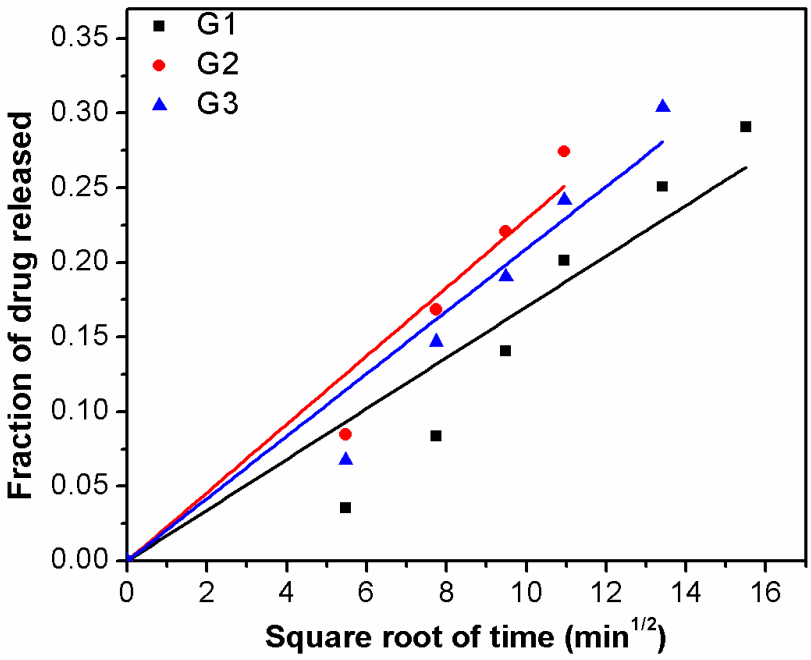

Figure 10: Higuchian kinetics of formulations

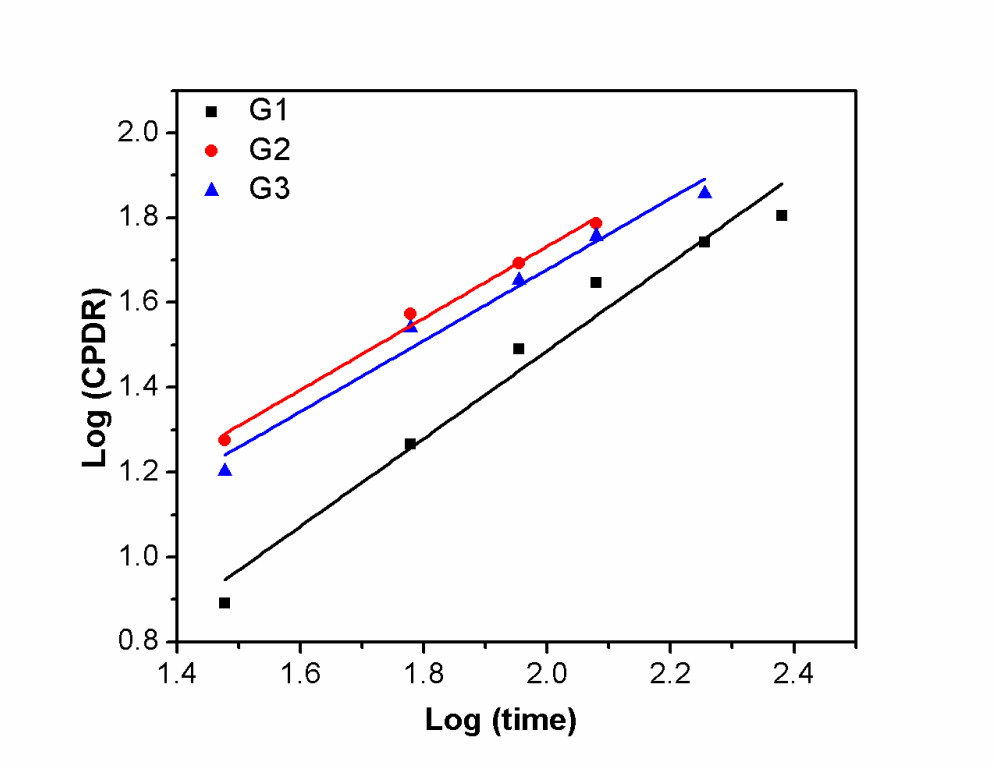

Figure 11 : KP kinetics of formulations.

Table 5: showing drug release values

Sample	CPDR	R2 for model fitting				Type of release
		Zero Order	Higuchi model	KP model		
				R^2	n-value	
G1D	45.43	0.928	0.895	0.970	0.86	Non-Fickian
G2D	44.82	0.777	0.928	0.991	0.84	Non-Fickian
G3D	42.28	0.806	0.946	0.979	0.83	Non-Fickian

4.8. Antimicrobial Assay

The antimicrobial efficacy of G1D, G2D and G3D against against *E.coli* was found to be very poor. The blank gel did not show any antimicrobial activity indicating that the incorporated drug was responsible for the observed zone of inhibition. The zones of inhibition for G1D, G2D and G3D were observed to be 2.1 cm, 1.7 cm and 1.1 cm respectively.

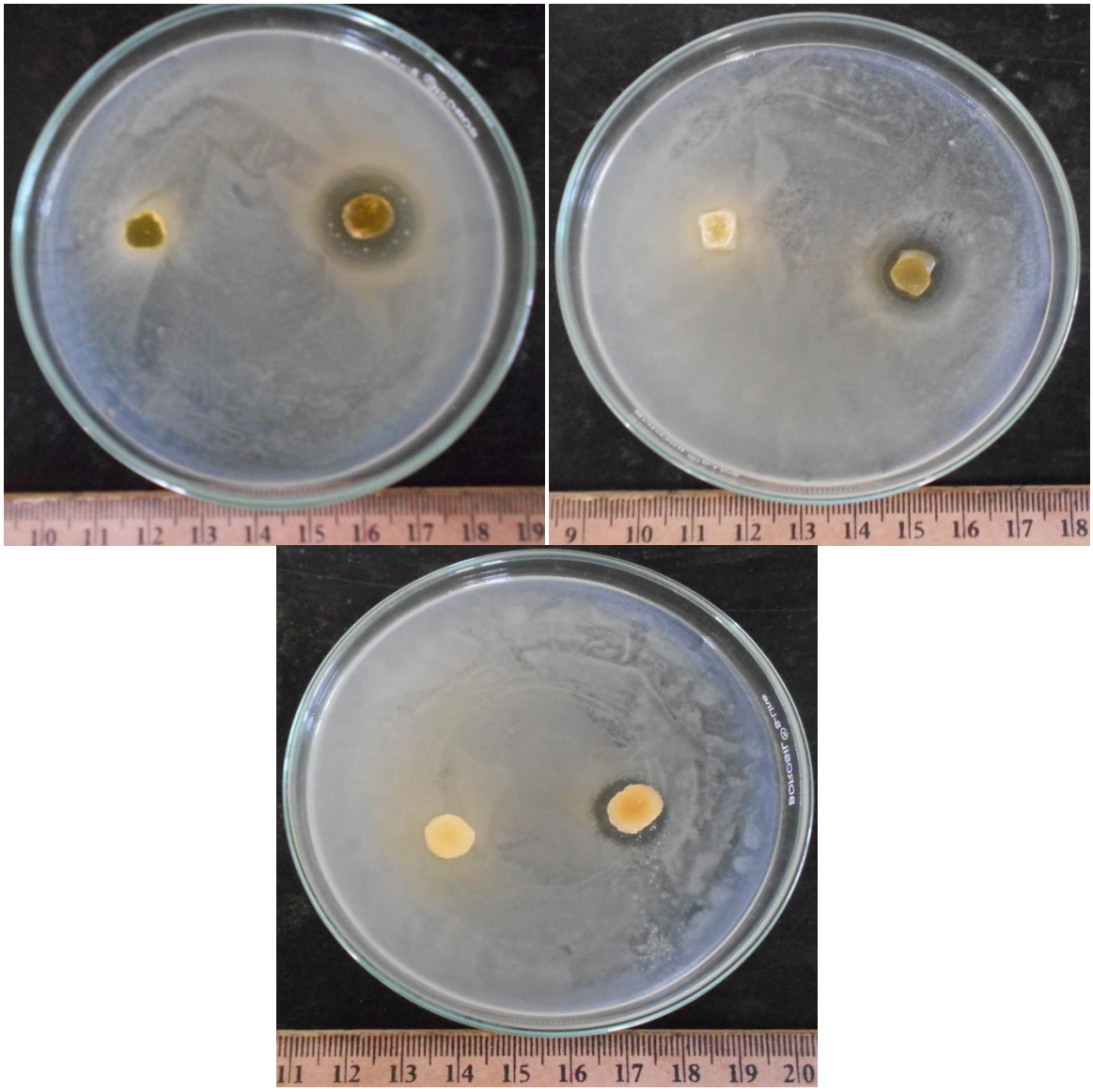

Figure 12: showing antimicrobial assay for G1D, G2D and G3D respectively.

Chapter 5
Conclusion

5. Conclusion

The study showed that the hydrogel, emulgel and bigel formulations were hemocompatible and highly mucoadhesive in nature.XRD study established the amorphous nature of the gel. The swelling behavior complements drug release studies and indicated that hydrogel formulation had higher release ability than emulgel and bigel formulations. The impedance study confirmed the conductivity of the gel preparations with higher conductivity in hydrogel formulation due to its higher aqueous content.

Chapter 6
Reference

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