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HYDROGELS FOR PHYTOCHEMICALS DELIVERY & WOUND HEALING APPLICATION

By

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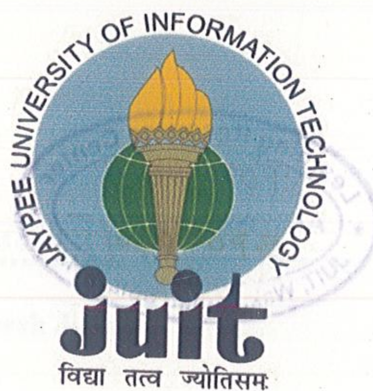

(MAY 2013)

Submitted in partial fulfillment of the Degree of

Bachelor of Pharmacy

DEPARTMENT OF BIOTECHNOLOGY, BIOINFORMATICS AND PHARMACY

JAYPEE UNIVERSITY OF INFORMATION TECHNOLOGY WAKNAGHAT

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CERTIFICATE

This is to certify that the work entitled "**Hydrogels for phytochemicals delivery and wound healing application**" submitted by **Ms. Disha Kulshrestha** and **Ms. Nidha amir,** in partial fulfillment for the award of Degree of Bachelor of Pharmacy of Jaypee University of Information Technology, Waknaghat (Solan), has been carried out under my supervision. This work has not been submitted partially or wholly to any other University or Institute for the award of this or any other degree or diploma.

Signature of Supervisor ....

Name of Supervisor **Dr. Maneesh Jaiswal**

Designation **Lecturer**

Date 28th May 2013

ACKNOWLEGEMENT

We are immensely thankful and express our heartfelt gratitude to project supervisor **Dr. Maneesh Jaiswal** without whom benign guidance and concrete advise, this project would not have seen the light of the day. We hold him in reverential awe.

We would like to thank **Mr. Arun Dhiman** for the encouragement and his constant interest in the activities of our project right from its inception.

We would like to acknowledge our hearty gratitude towards all teaching staff at Department of Pharmacy, JUIT, Waknaghat. They not only taught the fundamental essential for undertaking such a project but also helped us to develop individually. Without their guidance it would have been extremely difficult to grasp and visualize the project theoretically.

We are grateful to **Mrs. Sonica Gupta,** who has been a constant source of help in Pharmaceutical laboratory.

We would also like to thank **Ms. Shivani Sood** for helping us with the microbial studies carried out at Biotechnology lab at Jaypee University of Information Technology, Waknaghat.

We would also like to thank our friends in the Pharmacy department for their constructive criticism and encouragement. Last and certainly not the least, we are indebted to our family members for their unflinching support to us from the first day.

ABSTRACT

In this study, pH-sensitive poly (acrylamide)/chitosan hydrogels were synthesized as controlled release systems using chitosan as a matrix forming polymer, bisacrylamide as crosslinker and ammonium persulfate/sodium meta-bisulfite as initiators. A set of hydrogels were used in the form they were prepared. Prepared hydrogels were loaded with hydroalcoholic leaf extract of *Urtica diocia*. Positive effect of hydrogels on wounds and enhanced wound healing process has been proven. Hydrogels provide a warm, moist environment for wound that makes it heal faster in addition to their useful mucoadhesive properties. Moreover, hydrogels can be used as carriers of variety of drugs, such as antimicrobial drugs. This will provide a depot release of drug to the wound bed. Antibacterial activity for extract of *Urtica diocia and pinus roxburghii* was carried out againt E.coli, S.aureus and B. subtilis. Extracts showed positive result on S.aureus and B.subtilis. Hydrogels were evaluated for degradation and swelling % which came out to be 53%.

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CHAPTER 1
OBJECTIVE, INTRODUCTION AND REVIEW OF LITERATURE

OBJECTIVE OF THE STUDY

From the literature survey it has been found that certain phytochemicals e.g. Curcumin, vitamins, extract of neem fruit as well as leafs, tannins, calendula flower oil, *urtica dioca* and some amino acids have good antioxidant, anti-inflammatory, antimicrobial and healing promoting activity, therefore sustained delivery of these phytochemicals in a controlled manner at the wound site for a longer period of time could have a more realistic approach to get an effective therapeutic and multiple application system for wounds. To achieve above aim certain approaches has been applied and divided into following parts.

1. Synthesis and evaluation of polyacrylamide/chitosan hydrogels for it antioxidant and antimicrobial activities.
   a) Qualitative, quantitative estimation, antioxidant and antimicrobial activity of plant extract.
   b) Antioxidant, antimicrobial activity of extract loaded hydrogels.
   c) Physicochemical properties of hydrogels like swelling index, degradation rate

INTRODUCTION

1. Hydrogels

Hydrogels is a network of polymer chains that are hydrophilic, some time found as a colloidal gel in which water is the dispersion medium. Hydrogels are highly absorbent (they can contain over 99.9% water) natural or synthetic polymers. Hydrogels also possesses a degree of flexibility very similar to natural tissue, due to their significant water content. In general, hydrogels can be prepared from either synthetic polymers or natural polymers. The synthetic polymers are hydrophobic in nature and chemically stronger compared to natural polymers. Their mechanical strength results in slow degradation rate, but on the other hand mechanical strength provides the durability as well. These two opposite properties should be balanced through optimal design. Polymeric gels are the liquid-solid systems. It means that they have a solid matrix that swells in water and forms a three dimensional network. These polymers do not dissolve in the liquid. Creating this three dimensional network is a result of cross linking That is again a result of chemical bindings[1,2].

The main features of hydrogels influencing their use in wound treatment are:

1. Shape stability and softness similar to that of the soft surrounding tissues
2. Chemical and biochemical stability
3. Absence of extractable
4. High permeability for water-soluble nutrients and metabolites across the Biomaterial Tissue-interface.
5. Hydrogels can be the most suitable dressing in debridement stage of a chronic wound

Some additional advantages of hydrogels as wound dressings are:

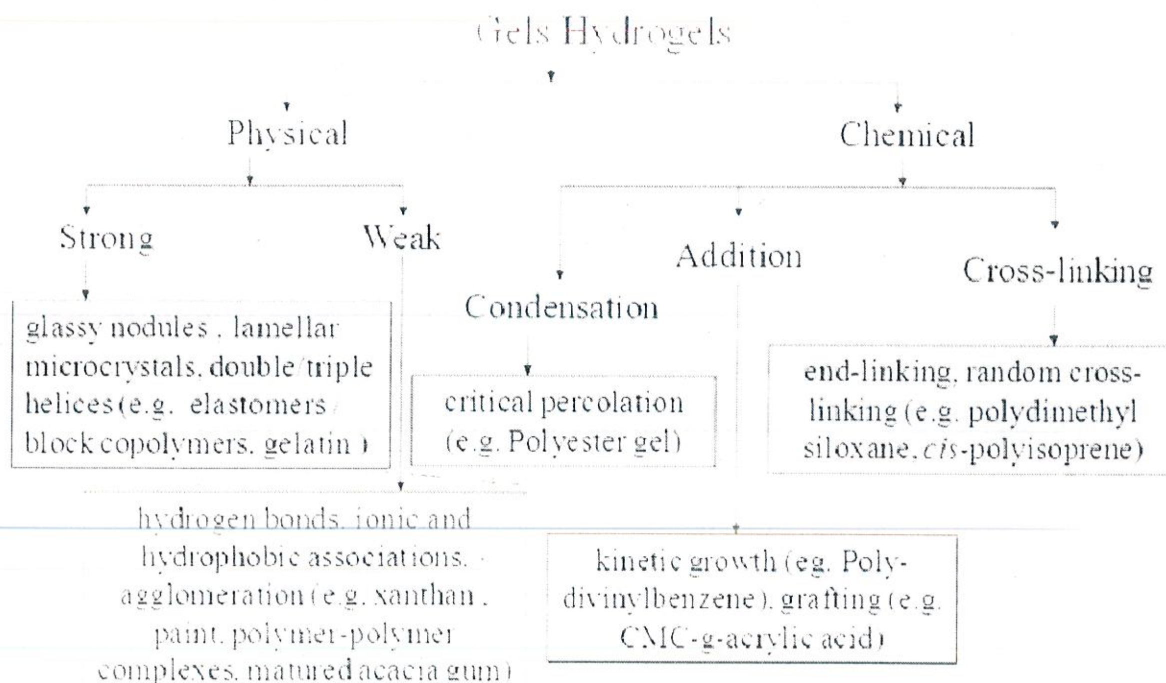
1. Suitable rheological properties
2. Good tissue compatibility
3. Convenience in handling
4. Ease of application
5. Excellent biocompatibility due to their high water content.

1.1 CLASSIFICATION OF HYDROGELS

Chemically cross-linked hydrogels: Radical polymerization is usually applied to make these polymers. When these types of hydrogels come in contact with H2O molecules, they begin to swell up and spread their network[3]. Physically cross-linked hydrogels: Physically cross-linked hydrogels do not need introduction of an external cross-linking agent. Cross-linking agents are usually nondegradable and can be toxic and a removal of their residuals may be needed before they can be used in biomedical or pharmaceutical purpose. The physically crosslinked hydrogels are usually biodegradable[4]. Their amorphous hydrophilic phase is held together by highly ordered aggregated chain segments held together by secondary molecular forces such as hydrogen bonding, Van der Waals forces or hydrophobic interaction. There are several other classifications for hydrogels. They can also be classified based on the nature of the network: homopolymer, copolymer, interpenetrating, or double networks; physical structure: homogeneous (optically transparent), microporous, and macroporous hydrogels; or in relation to their fate in the organism: degradable and nondegradable hydrogels[5].

Classification of hydrogels

Figure 1:- Flow chart on classification of hydrogels



Taken from:- Progress in Molecular and Environmental Bioengineering- From Analysis and Modeling to Technology Applications

1.2 Biomedical applications of Hydrogels

Hydrogels that are used for biomedical purposes should be biocompatible and often biodegradable. Drug delivery: right after hydrogels were discovered, their use as anticancer and antibiotic deliver systems was studied. Hydrogels have a porous network. Often we can control the porosity of hydrogels by controlling the density of cross-links or by changing the swell affinity of hydrogels in the environment. This porosity property of hydrogels helps the release of drugs from hydrogels. The release of drug from hydrogels can be controlled by controlling the diffusion coefficient of drugs through hydrogel matrix. We can also make a depot formulation of hydrogel-drug. The depot formulation can be made by trapping drugs into the hydrogel. Hydrogels for tissue engineering and regenerative medicine: hydrogels enable the incorporation of growth factors and control over their release. The release rate is controlled by degree of cross-linking of hydrogel. The protein will diffuse out of hydrogel through the water pathways. Gelatin hydrogel was able to release the incorporated growth factor for up to 3 months(6,7).

There are many kinds of tissue engineering scaffolds. Among all of these hydrogels are the most popular candidates due to:-

- Their structure.
- Their good biocompatibility
- Tunable viscoelasticity
- High water content
- High permeability of oxygen
- Essential nutrients

1.3 Methods to produce hydrogels

1.3.1 Phsical crosslinking:- There has been an increased interest in physical or reversible gels due to relative ease of production and the advantage of not using cross-linking agents. These agents affect the integrity of substances to be entrapped (e.g. cell, proteins, etc.) as well as the need for their removal before application.(7,8)

The various methods reported in literature to obtain physically cross-linked hydrogels are:-

I. **Heating/cooling a polymer solution:-**Physically cross-linked gels are formed when cooling hot solutions of gelatine or carrageenan. The gel formation is due to helix-formation, association of the helices, and forming junction zones. Carrageenan in hot solution above the melting transition temperature is present as random coil conformation. Upon cooling it transforms to rigid helical rods.

II. **Ionic interaction:-** Ionic polymers can be cross-linked by the addition of di- or tri-valent counterions. This method underlies the principle of gelling a polyelectrolyte solution with a multivalent ion of opposite charges.

III. **Complex coacervation:-** Complex coacervate gels can be formed by mixing of a polyanion with a polycation. The underlying principle of this method is that polymers with opposite charges stick together and form soluble and insoluble complexes depending on the concentration and pH of the respective solutions.

IV. **H-bonding:-** H-bonded hydrogel can be obtained by lowering the pH of aqueous solution of polymers carrying carboxyl groups.

V. **Freeze thaw method:-** Physical cross-linking of a polymer to form its hydrogel can also be achieved by using freeze-thaw cycles. The mechanism involves the formation of microcrystals in the structure due to freeze-thawing.

VI. **Free radical copolymerization:-** is a method of polymerization by which a polymer forms by the successive addition of free radical building blocks. Free radicals can be formed via a number of different mechanisms usually involving separate initiator molecules. Following its generation, the initiating free radical adds (nonradical) monomer units, thereby growing the polymer chain. Free radical polymerization is a method of polymerization by which a polymer forms by the successive addition of free radical building blocks. Free radicals can be formed via a number of different mechanisms usually involving separate initiator molecules. Following its generation, the initiating free radical adds (nonradical) monomer units, thereby growing the polymer chain.

2. Wounds

A wound is any type of injury to the skin. Wounds can be open wounds, in which the skin is broken or torn or closed wounds. Although open wounds can bleed and run the risk of infections, closed wounds can also be dangerous depending on the extent of tissue damage. There are five major categories of wounds; each is distinctive in its appearance and the source of the injury.[16,17]

2.1 Types of Wounds:-

I. Incision

An incision wound is a cut in the skin caused by a sharp object such as a knife, broken glass, scissors or surgeon's scalpel. Incision wounds are 'neat' and the edges of the skin are usually smooth.

II. Laceration

A laceration is injury to the skin that results in the skin being cut or torn open, as described by the National Institutes of Health. Lacerations can be shallow, only injuring the surface skin, or deep, causing injury to the muscles, tendons, ligaments, blood vessels or nerves. Lacerations are most commonly made by some sort of blunt trauma such as being hit with a fist or baseball bat. The difference between an incision wound and a laceration wound, according to the Biomedical Journal, is that a laceration is generally jagged, since the skin is torn instead of cut.

III. Abrasion

An abrasion is a type of wound in which the skin is scraped or rubbed off. When skin is dragged on carpet, the resulting wound, often called a carpet burn, is an abrasion. Abrasions are usually superficial wounds, meaning that only the outer layers of skin are affected. A deep abrasion, one that penetrates to the inner layers of skin, can leave a scar. Parts of the body with thin layers of skin, such as the knees and elbows, are most prone to abrasions according to the State of Victoria.

IV. Contusion

A contusion is a kind of closed wound, meaning that the skin is not broken. Contusions are caused by blunt force trauma to the skin that results in tissue damage. When the blood vessels under the skin are broken, blood pools under the skin causing a bruise.

V. **Puncture**

A puncture wound is created when a sharp object enters the skin. These wounds are usually small and do not bleed a lot. Although these wounds tend to close over quickly, according to doctors at the Mayo Clinic, they still need treatment as infection is a possibility. Puncture wounds are prone to a tetanus infection, according to the State of Victoria, so it is important to seek medical advice for any puncture wound. Common types of puncture wounds include stepping on a nail or bites from animals.[17]

2.2 Wound healing process: -The wound healing process is a series of independent and overlapping stages. In these stages will both cellular and matrix compounds work to reestablish the integrity of damaged tissue and replacement of lost tissue[18]. These overlapping series can be classified in five stages:

Haemostasis: The first response to injury is bleeding. Bleeding is an effective way to wash out bacteria that are on the surface of skin. Afterwards, bleeding activates haemostasis stage that is initiated by clotting factors. The clot dries out and creates a hard surface over the wound that protects tissues underlying.

Inflammation: This stage starts almost at same time as haemostasis. It occurs from between few minutes to up to 24 minutes after injury. In this stage histamine and serotonin are released into wound area and activate phagocytes to enter the wound area and engulf dead cells.

Migration: In this stage the reestablishment of wound begins. The epithelial cells and fibroblasts move into the injured area and grow rapidly under the hard scab to replace the damaged tissue.

Proliferation: this stage has three characteristics. First, the granulation tissue is formed by growth of capillaries. Second, lymphatic vessels enter into wound and the third one, synthesis of collagen starts providing form and strength to the injured tissue.

Maturation: in this stage, the shape of the final scar is determined by formation of cellular connective tissue and strengthening of the new epithelium. The different stages in wound healing process can be defined in simplified form as

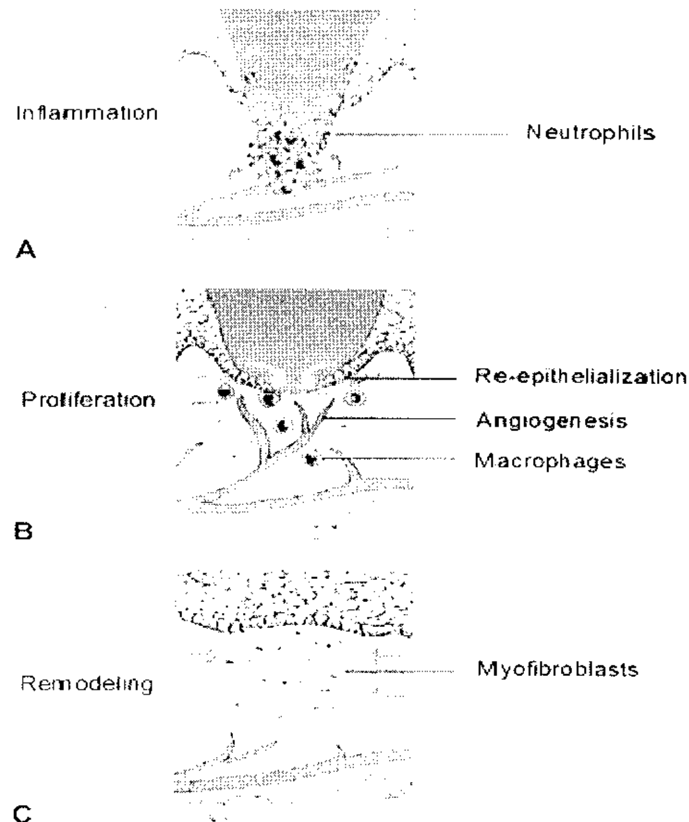


Figure 2:- Wound healing process

Taken From:- M.A. Fonder, Treating the chronic wound: A practical approach to the care of nonhealing wounds and wound care dressings. ***Journal of the American Academy of Dermatology*** **58, 185-206.**

An ideal material to be applied to wound should be:-

- Nontoxic
- Biocompatible
- Enhance cellular interaction and tissue development
- Be biodegradble and bioresorbable

The ideal properties of a wound dressing are:-

- Providing a moist environment
- Creating a protective mechanical barrier and thermal isolation

- Protecting against secondary infections
- Keeping the wound environment moist
- Absorbing the exudates and bacteria
- Promoting debridement
- Contributing to simple gas exchange
- Decreasing or removing trauma in the defected area
- Being acceptable for patient
- Not possessing any toxic, irritant or allergic properties
- Cost-effectiveness

3. Plants used

3.1 *Urtica diocia*

Common nettle (Urtica dioica L), a herbaceous perennial flowering plant, is a member of the Urticaceae family. Traditional herbal medicine in the Balkan countries uses stinging nettle leaves in the form of an herbal infusion as a remedy for the treatment of diarrhea, vaginal discharge, internal/external bleeding. Being rich in chlorophyll, nettle leaves are used for the treatment of anemia as well as general well-being, and more recently as natural food colorant. The stinging nettle leaf contains chlorophyll, vitamin C, vitamin K, panthotene acid, carotenoids, B group vitamins (B1 and B2), tannins, essential oil, proteins, and minerals (Fe, Cu, Mn and Ni).Stinging nettle hairs contain acethylcholin and histamine while the stem and root contain flavonoids .Animal studies proved that nettle leaf extract may inhibit blood clotting (platelet aggregation), can decrease total cholesterol levels as well as enhance the overall liver function. Water extract of stinging nettle makes significant inhibition of adenosine deaminase activity in prostate tissue in the patients with prostate cancer. Adding dried powder of nettle into laying hens diets significantly increases egg production, proves the modulating effects of the immune parameters , and lowers the total cholesterol and triglycerides concentration. It has also been reported that the stinging nettle extract exibits antioxidant, antimicrobial, antiulcer, and analgesic activities.

3.2 *Pinus roxburgii*

Pinus roxburghii Sarg. has many medicinal uses, the wood is aromatic, deodorant, haemostatic, stimulant, anthelmintic, digestive, liver tonic, diaphoretic, and diuretic. It is useful in eye, ear, and pharynx diseases, foul ulcers, haemorrhages, haemoptysis, worn infections, flatulence, liver diseases, bronchitis, inflammations, skin diseases, pruritus, and giddiness.The chief chemical constituents of turpentine oil from Pinus roxburghii Sarg. are α-pinene, β-pinene, car-3-ene and longifolene hydrocarbons (d- and l-pinene), resin acids, camphene, fenchene, dipentene, and polymeric terpenes.

Extraction process:-[19,20]

A Soxhlet extraction is only required where the desired compound has a *limited* solubility in a solvent, and the impurity is insoluble in that solvent. If the desired compound has a significant solubility in a solvent then a simple filtration can be used to separate the compound from the insoluble substance.

Fruit extraction in progress. The sample is placed in the thimble.Normally a solid material containing some of the desired compound is placed inside a thimble made from thick filter paper, which is loaded into the main chamber of the Soxhlet extractor. The Soxhlet extractor is placed onto a flask containing the extraction solvent. The Soxhlet is then equipped with a condenser.

The solvent is heated to reflux. The solvent vapour travels up a distillation arm, and floods into the chamber housing the thimble of solid. The condenser ensures that any solvent vapour cools, and drips back down into the chamber housing the solid material.

The chamber containing the solid material slowly fills with warm solvent. Some of the desired compound will then dissolve in the warm solvent. When the Soxhlet chamber is almost full, the chamber is automatically emptied by a siphon side arm, with the solvent running back down to the distillation flask. The thimble ensures that the rapid motion of the solvent does not transport any solid material to the still pot. This cycle may be allowed to repeat many times, over hours or days.

During each cycle, a portion of the non-volatile compound dissolves in the solvent. After many cycles the desired compound is concentrated in the distillation flask. The advantage of this system is that instead of many portions of warm solvent being passed through the sample, just one batch of solvent is recycled.

After extraction the solvent is removed, typically by means of a rotary evaporator, yielding the extracted compound. The non-soluble portion of the extracted solid remains in the thimble, and is usually discarded.

LITERATURE SURVEY

Stanford

The antimicrobial hydrogel wound dressing is a swellable polymer gel made from about 7-9% (wt/vol) polyvinyl alcohol (PVA), preferably 8.9%, about 0.1% (wt/vol) polyvinyl pyrrolidone (PVP), and about 1-2% (wt/vol) agar, preferably 1%, the balance (about 90%) being distilled water, the foregoing contents being crosslinked by gamma radiation at a dose of about 30 kGy. Prior to crosslinking by gamma radiation, an effective amount of a pair of antibiotics is added to the gel at room temperature. The antibiotics include about 10,000 IU of polymyxin B sulfate, and about 5 mg neomycin per gram of gel. The polymyxin provides effective protection against various forms of gram negative microorganisms, and the neomycin is a broad spectrum antibiotic that provides protection against various forms of gram positive microorganisms. The hydrogel has sufficient mechanical strength for use as a wound dressing, and is capable of absorbing water up to 900% of its volume.

Zohidi et al. prepared a novel cross-linked honey hydrogel dressing by incorporating Malaysian honey into hydrogel dressing formulation, cross-linked and sterilized using electron beam irradiation . In this study, the physical properties of the prepared honey hydrogel and its wound healing efficacy on deep partial thickness burn wounds in rats were assessed. Application of honey hydrogel dressings significantly enhanced ($P < 0.05$) wound closure and accelerated the rate of re-epithelialization as compared to control hydrogel and opposite film dressing. A significant decrease in inflammatory response was observed in honey hydrogel treated wounds as early as 7 days after burn ($P < 0.05$).

Enaida et al. showed that curcumin, a lipophilic compound found in the plant *Curcum longa* L., exhibits a wide range of pharmacological activity; however, its therapeutic use has been limited because of its low bioavailability following oral administration. The aim of this study was to evaluate the viscoelastic characteristics and biocompatibility of a curcumin/xanthan:galactomannan hydrogel (X:G) system after topical application on chick embryo chorioallantoic membrane (CAM), a system established with a view

toward curcumin nasal or topical pharmaceutical applications or possible administration in cosmetics or foods. A rheological analysis indicated that incorporation of curcumin did not alter the viscoelastic characteristics of the X:G hydrogel, suggesting that there was no change in the structure of the gel network. X:G hydrogels did not induce CAM tissue injury and the curcumin/X:G hydrogel system was also highly biocompatible.

Bahadoor et.al showed that application of hydrogels on wounds enhances the wound healing process. Hydrogels provide a warm, moist environment for wound that makes it heal faster in addition to their useful mucoadhesive properties. Characterizing the behaviour of Carbopol hydrogels by texture analyser as described in this Thesis is a new method. As a first step, we evaluated the effects of each parameter used in texture analysis in order to establish reproducible measuring conditions. Behaviour of Carbopol Ultrez 10 hydrogels was studied in regard to the polymer concentration and pH of the gel. Accelerated stability tests were conducted for both Carbopol gels and liposomal Carbopol gels. Chloramphenicol was used as a drug model to be entrapped in liposomes and its release from liposomal hydrogels was studied Accelerated stability indicated that the attention needs to be given to the gel composition. When incorporating liposomal dispersions, there is a limit of proportion of the dispersion which could be incorporated without affecting the original properties of the gel. Liposomal Carbopol hydrogels show potential to be used as drug delivery system.

Pinardag et al synthesize pH-sensitive poly(acrylamide-co-acrylic acid) hydrogels in the presence of N,N-methylene bisacrylamide as crosslinker and ammonium persulfate as initiator. A set of hydrogels were used in the form they were prepared. One set of hydrogels were prepared as porous networks by incorporating sodium chloride into the reaction medium and then leaching of it after the completion of polymerization reaction. Two sets of hydrogels were modified by argon-plasma at different discharge powers. Hydrogels were characterized by 13C-NMR, XPS, SEM, ATR-FTIR, ESR as well as equilibrium degree of swelling (EDS) and contact angle measurements. Prepared hydrogels were loaded with a model antibiotic, ciprofloxacin-HCl (CPFX), and in-vitro release of CPFX from hydrogel matrices were examined in buffer solutions of varying pH values. There are two factors determining the release rates of CPFX; one is the pH-dependent solubility of CPFX and the other is EDS of the hydrogel samples. For porous

samples drug loading and release rates were higher when compared to the control samples and CPFX solubility dominated over release kinetics. Plasma treatment resulted in prolonged release rates in acidic medium.

Byasal K.et.al Chitosan hydrogels may be formed by various mechanisms. In this study, we aimed to form hybrid polymer networks of chitosan with alginate using a crosslinker which enabled the covalent binding of the two macromolecules The swelling properties of these gels were analyzed in water and in phosphate buffered saline (PBS) solution. The presence of alginate in a chitosan/alginate hydrogel was shown to support the hydrogel stability. Compared to chitosan/alginate (1/2) hydrogel prepared with 1wt% DCC, the swelling of chitosan/alginate (1/2) hydrogels prepared with 3wt% DCC was limited. To measure the degree of cell proliferation, the hydrogels were seeded with L929 mouse fibroblasts and cell numbers measured by neutral red uptake assay. The cell attachment was also followed by (SEM) photography. It was observed that chitosan/alginate (1/2) hydrogels with 1wt% (DCC) provides a better environment for cell attachment and proliferation. This study presents functional hydrogel formation by crosslinked chitosan and alginate, a novel biomaterial which also supports cell growth.

Riberio M.P.et.al In the present study in vitro and in vivo assays were carried out to evaluate the applicability of a dextran hydrogel loaded with chitosan microparticles containing epidermal and vascular endothelial growth factors, for the improvement of the wound healing process.. The wound healing process was monitored through macroscopic and histological analysis. The macroscopic analysis showed that the period for wound healing occurs in animals treated with microparticle loaded hydrogels containing growth factors that were considerably smaller than that of control groups. Moreover, the histological analysis revealed the absence of reactive or granulomatous inflammatory reaction in skin lesions. The results obtained both in vitro and in vivo disclosed that these systems and its degradation by-products are biocompatible, contributed to the re-establishment of skin architecture and can be used in a near future for the controlled delivery of other bioactive agents used in regenerative medicine.

Pulat M.et al. The aim of this study is to prepare a novel wound dressing material which provides burst release of an antibiotic in combination with sustained release of growth factor delivery. This might be beneficial for the prevention of infections and to stimulate wound healing. As a wound dressing material, the semi-interpenetrating network (semi-IPN) hydrogel based on polyacrylamide (PAAm) and chitosan (CS) was synthesized via free radical polymerization. Ethylene glycol dimethacrylate was used for cross-linking of PAAm to form semi-IPN hydrogel. The hydrogel shows high water content (~1800%, in dry basis) and stable swelling characteristics in the pH range of the wound media (~4.0-7.4). The antibiotic, piperacillin-tazobactam, which belongs to the penicillin group was loaded into the hydrogel. The therapeutic serum dose of piperacillin-tazobactam for topic introduction was reached at 1st hour of the release. The successful sustained release behavior of CS-PAAmhydrogel for EGF maintained the presence of EGF in the culture up to 5 days and the highest mitochondrial activities were recorded for the 0.4 μg EGF-loaded/mg of hydrogel group.

CHAPTER 2
MATERIALS AND METHOD

MATERIALS AND METHODS

Table 1:- Materials used during the formulation of hydrogels

Sr.No.	Materials
1	Acrylamide
2	Bis-acrylamide
3	Chitosan
4	Sodium metabisulphite
5	Aluminium persulphate
6	Ethanol
7	Methanol
8	Glacial acetic acid
9	Sodium hydroxide
10	Folin reagent
11	Ferric chloride
12	Gallic acid
13	Quercetin
14	Sodium carbonate
15	Tannic acid
16	Sodium nitrite
17	Ammonia solution
18	Sulphuric acid
19	Sodium chloride
20	Potassium dihydrogen phosphate
21	Potassium hydrogen phosphate
22	Sodium alginate
23	di- methyl sulphoxide
24	Polyvenyl alcohol
29	DPPH

Table 2:- Equipments used

Sr. No	Equipments
1	Spectrophotometer
2	Autoclave
3	Laminar floor
4	Soxhlet apparatus
5	Rota evaporator
6	Incubator
7	Refrigerator of 4°, -80°
8	Incubator
9	Pipette tips
10	Mico-pipette

4. Formulation of hydrogel using free radical copolymerization method

Free radical polymerization is a method of polymerization by which a polymer forms by the successive addition of free radical building blocks. Free radicals can be formed via a number of different mechanisms usually involving separate initiator molecules. Following its generation, the initiating free radical adds (nonradical) monomer units, thereby growing the polymer chain. Free radical polymerization is a method of polymerization by which a polymer forms by the successive addition of free radical building blocks. Free radicals can be formed via a number of different mechanisms usually involving separate initiator molecules. Following its generation, the initiating free radical adds (nonradical) monomer units, thereby growing the polymer chain.

In the formulation of hydrogel we added Aluminium persulfate and metabisulfite as the initiator. Crosss linking was done between chitosan,acrylamide and bisacrylamide.

Table 3:- Formulation table of hydrogels

Chemicals	Amount (weight in ratio)
Acrylamide	10.56 moles
Chitosan	2%, 3%, 4% w/v
Bis acrylamide	1% mole % of acrylamide
Aluminium persulphate	0.5%mole % of acrylmaide
Sodium metabisulphite	0.5% mole % of acrylamide

5. Quantitative estimation of Chemical constituent

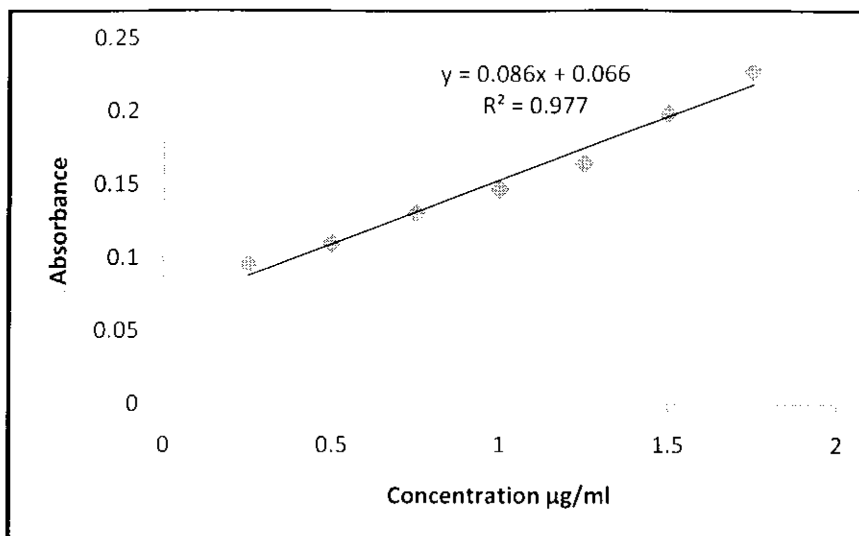
a. Determination of total phenols by spectrophotometric method:

To analyze the total phenolic content (TPC), the method of **Kim et al. (2003)** was used to make the Folin Ciocalteu reagent. For 0.2 ml of the extract (prepared in 70% ethanol with a concentration of 1.0 mg/ml), 0.4 ml of Folin-Ciocalteu reagent was mixed and the solution was allowed to stand at 25°C for 5 to 8 min before adding 0.2 ml of 7.0% sodium carbonate solution. Using deionized water, the final volume was made to 10.0 ml. After two hours, absorbance was measured at 765 nm. Thus, the calibration curve was drawn using gallic acid as standard for total phenolics (TPC) which was measured as mg gallic acid equivalents (GAE) per gram of the sample (mg/g)[21].

Table 4:-Calibration curve for Gallic acid

Concentration µg/ml	Absorbance
0.25	0.0952
0.50	0.1098
0.75	0.1302
1.0	0.1462
1.25	0.1641
1.50	0.1982
1.75	0.2275

Figure 3:- Calibration curve for Gallic acid

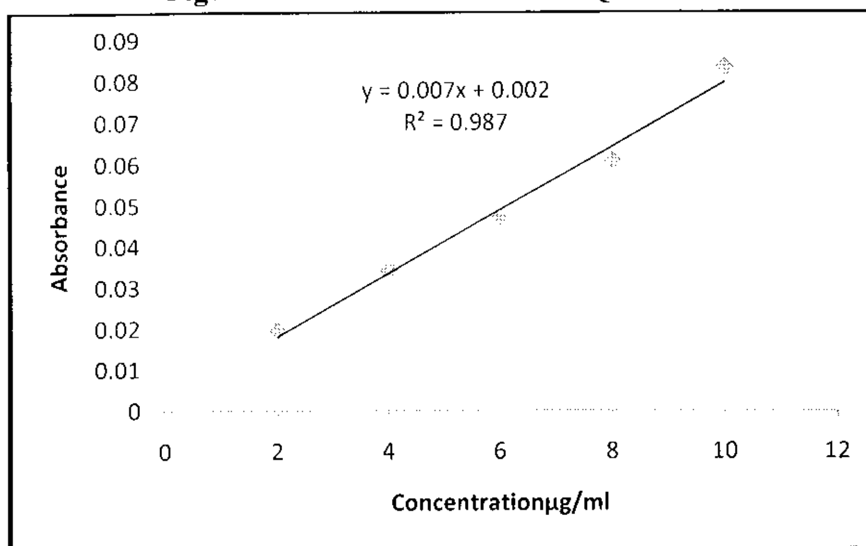


b. **Determination of total Flavonoids:-** For the assessment of flavonoids, the colorimetric method introduced by **Dewanto et al. (2002)** was adapted. To determine the amount of flavonoids by the aforementioned method, 1.50 ml of deionized water was added to 0.25 ml of the sample extract from stock solution (concentration of 1 mg/ml of methanol), followed by 90 µl of 5% sodium nitrite (NaNO2). Six minutes later, after addition of 180 µl of 10% AlCl3, the mixture was allowed to stand for another 5 min before addition of 0.6 ml of 1 M NaOH. By adding deionized water and mixing well, final volume was made up to 3 ml. Thus, the calibration curve was drawn using quercetin as standard for total flavanoids at 510nm[21].

Table 5:-Calibration curve for Quercetin

Concentration µg/ml	Absorbance
2	0.0197
4	0.0343
6	0.0469
8	0.0812
10	0.0935

Figure 4:-Calibration curve for Quercetin



c. **Determination of Tannin content:** - 500 mg of plant sample was weighed and transferred to 50 ml flask. Then added 50 ml of distilled water and stirred for 1 h. Sample was filtered into a 50 ml volumetric flask and the volume was made up to the mark. 5 ml of the filtered sample was pipette into test tube and then mixed with 2 ml of 0.1 M ferric chloride. The absorbance was measured using spectrophotometer at 395 nm wavelength within 10 min. Thus, the calibration curve was drawn using tannic acid as standard at 395nm.[21]

Table 6:-Calibration curve for tannic acid

Concentration µg/ml	Absorbance
0.02	0.213
0.04	0.388
0.06	0.645
0.08	0.798
0.10	0.996

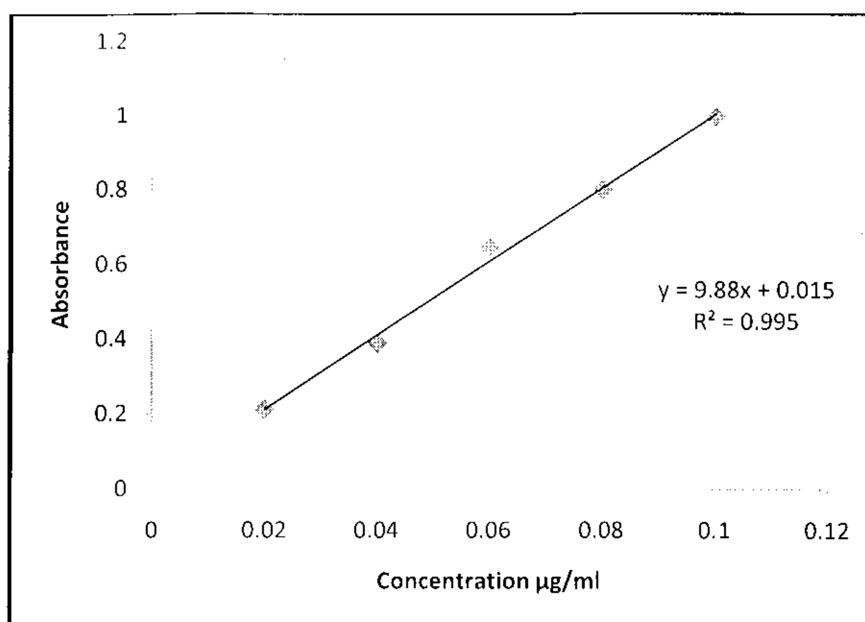


Figure 5:- Calibration curve for tannic acid

6. **Qualitative tests for phytochemical constituents:-**
   a. **Test for tannins:-**

Ferric chloride test: - Take 20ml of extract and add few drops of 0.1% ferric chloride solution. Brownish green or blue black colour determines the presence of tannin.

   b. **Test for flavanoids:** -

Alkaline reagent test: - To the test solution add few drops of sodium hydroxide solution, intense yellow colour is formed which turns to clourless on addition of few drops of dilute acid indicate presence of flavanoids.

   c. **Test for saponins:** -

Froth formation test: - place 2ml solution of drug in water in a test tube, shake well. Stable froth (foam) is formed[22]

7. **Antioxidant activity of plant extract:-**

a. **Determination of antioxidant property by the DPPH test:** - The DPPH test is based on the ability of the stable 2,2-diphenyl-1-picrylhydrazyl free radical to react with hydrogen donors. The DPPH• radical displays an intense UV-VIS absorption spectrum. In this test, a solution of radical is decolorized after reduction with an antioxidant (AH) or a radical

(R•) in accordance with the following scheme: DPPH• + AH → DPPH•-H + A•, DPPH• + R• → DPPH•-R. This method is very simple and also quick for manual analysis.

Reagent preparation: 0.95 mmol·L-1 solution of radical DPPH• (m = 0.00374 g/100 mL). First, this amount is dissolved in 50 mL of DMSO and after dissolution made up to a volume of 100 mL with ACS water. The solution can be used for 7 days when stored at 4 °C and in the dark.

Measurement procedure for an automated analyzer: A 200 μL volume of reagent is incubated with 20 μL of sample. Absorbance is measured after 1,520 seconds at λ = 510 nm. To calculate the antioxidant activity, the values determined before decrease of the absorbance (224th second of measurement – A224) and the last measurement value (1520th second of measurement –A1520) are used. Resulting value is calculated in accordance with the following formula[23,24].

Table 7:-Ascorbic acid DPPH scavenging activity

Conc(μg/ml)	Absorbance	Percentage inhibition
0.5	0.0306	50.56%
1.0	0.0243	60.74%
1.5	0.0179	71.08%
2.0	0.0160	74.1%
2.5	0.0137	77.8%
3.0	0.0087	85.9%

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Figure 6 :- Ascorbic acid acid DPPH scavenging activity

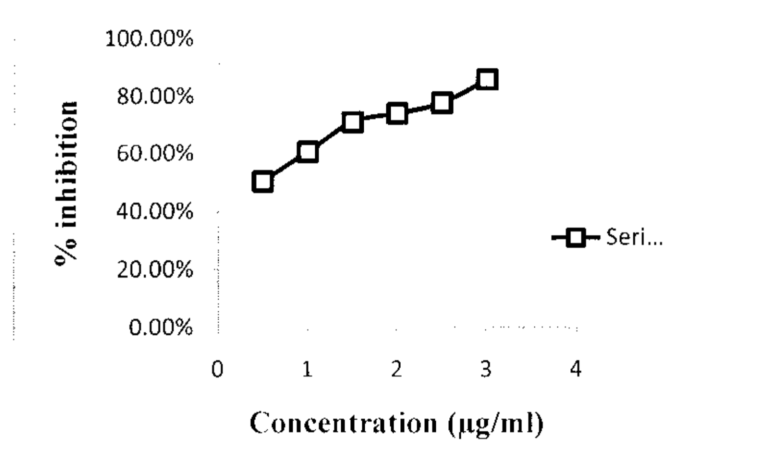
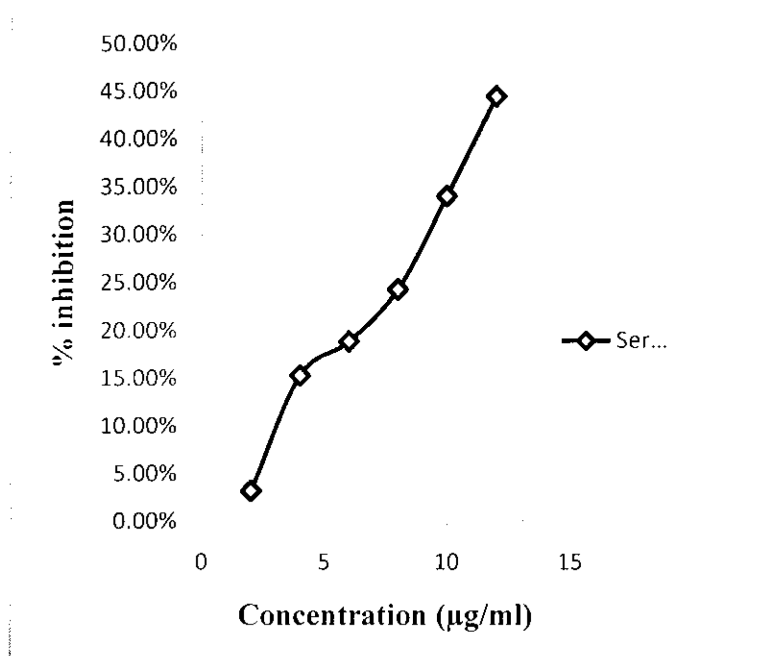


Table 8:-Urtica diocia DPPH scavenging activity:-

Conc(μg/ml)	Absorbance	Percentage inhibition
2	0.0559	3.2%
4	0.0452	15.31%
6	0.0502	18.9%
8	0.0468	24.3%
10	0.0408	34%
12	0.0344	44.4%

Figure 7:-:-Urtica diocia DPPH scavenging activity:-



8. Antimicrobial activities of plant extract (*Urtica dioca*)

8.1 Zone of Inhibition: - Antimicrobial agents are chemicals that are used against bacteria. There are many such agents available. Because there are many different situations where bacterial control is important, no antimicrobial agent is effective in all situations. For example, you wouldn't use the same compound to fight an ear infection as you would use to sterilize surfaces in an operating room. The situations are completely different. In one case, you are trying to assist the body to fight off an internalinfection, and in the other case, you are trying to eliminate bacteria from inanimate surfaces. You can use this method to compare the effectiveness of different disinfectants or different antibiotics against a strain of bacteria. Since this method depends on diffusion of the compound, it is important to keep several factors constant when you make your comparisons, including:

The size of the filter disks, the temperature of incubation, the composition and thickness of the agar and the uniformity of bacterial plating.

Materials and Equipment: - To do this experiment you will need the following materials and equipment, 6 nutrient agar plates:

- 3 plates will serve as controls, with no disinfectants,
- 3 plates will serve as test plates, with disinfectant disks.
- live E. coli culture (commonly available in labs; can also be purchased from online suppliers), sterile swabs, sterile tube with 10 ml sterile water, filter paper, hole punch, forceps, permanent marker, disinfectants (up to six), here are some ideas for different compounds to test:

Preparing Plates for Disk Diffusion Test

For this experiment, it is important to innoculate the plate with a uniform distribution of bacterial colonies, and to use the exact same procedure for each plate. Here are the steps for innoculating the control and test plates.

1. Prepare sterile filter disks by using a hole punch to make small circular disks from filter paper. You can use pencil or permanent marker to label each disk with a code for the disinfectant to be used for that disk (up to six). Keep track of the codes in your lab notebook. Wrap disks in aluminum foil and sterilize in a 300° oven for 30 minutes.
2. Use a permanent marker to mark the bottoms of the three test plates with as many sections as you have disinfectants (up to six). The sections should all be equal in size. Number the sections sequentially.
3. Label the three control plates. The purpose of these plates is to show that the bacteria consistently grow uniformly over the plate in the absence of disinfectant disks—confirming that your inoculation technique is consistent, and that the plates support uniform bacterial growth.
4. Prepare a dilute solution of bacteria for innoculating the plates.

a. Wipe a sterile swab across the surface of an existing plate (prepare from your E. coli culture 24 hours in advance).

b. Using proper sterile technique, open the tube of sterile water and swirl the swab in the water.

c. Cap the tube.

d. Properly dispose of the contaminated swab. Agitate the tube before using.

5. To inoculate a plate, dip a sterile swab in the dilute bacterial solution, using proper sterile technique. Gently wipe the swab over the surface of the plate, swabbing in three directions (120° apart) to insure complete coverage of the plate. Cover the plate and wait at least five minutes for the plate to dry.
6. Hold a single sterile disk by the edge with sterile forceps and dip it into the disinfectant solution to be tested (make sure it matches with the label on the disk). Touch the disk against the side of the container to drain off excess liquid.
7. Use sterile forceps to place a single disinfectant disk in the center of each of the marked sections on your test plates. Use the forceps to gently press each disk against the agar surface to insure good contact. Remember to use the exact same technique for each disk—consistency is very important for this experiment. Take notes in your lab notebook to keep track of which disinfectant is tested in each numbered section.
8. Incubate all of the plates, inverted, (agar on top) overnight. Use a longer incubation time if necessary (for example, for incubation at lower temperature).

Zones of Inhibition

1. After overnight incubation, examine your plates (keep them covered at all times).

a. The control plates should show uniform colonies over the entire surface of the plate. If the distribution is highly uneven, you will need to improve your inoculation technique and repeat the experiment.

b. If your disinfectants are effective at the concentrations you tested, you should see zones of inhibition around the disinfectant disks. The clear zones around each disk should have a uniform diameter, since diffusion of the compounds through the agar should be uniform in every direction. If this is not the case, suspect either your impregnation technique, or poor contact of the filter paper with the agar.

2. Measure the diameter of the zone of inhibition around each disk. Keeping the lid of the plate in place, use a ruler to measure the diameter of the clear area in millimetres. You will get three separate measurements for each disinfectant, one from each of the three test plates.

3. Are the diameters consistent across all three plates? Calculate the average and the standard deviation of the diameter of the zone of inhibition for each disinfectant.

8.2 Colony forming Unit (CFU)

Serial Dilutions

All three bacterial plate count methods described in lab require you to serially dilute your samples until you have 30-300 colony forming units (CFU) on the plate. Plates with more than 300 CFU are very difficult to count. Plates with less than 30 CFU are not statistically reliable. If you can see turbidity in a broth culture you have millions more bacteria than you need. If you plated straight from this turbid broth, all the CFU would grow together into a confluent mass. You would not be able to distinguish one colony from another. Traditionally serial dilutions were done with 1mL pipettes and 99mL sterile dilution bottles because they were the most accurate measuring devices available. With the advent of accurate and reliable micropipettes, smaller volumes can be used (0.1mL in 9.9mL or less of diluent). The important part of both methods is to ensure there is adequate mixing of your dilutions. In this lab we will be using water as the diluent. It is cheap and the organisms we use survive fine in it. Alternatively, phosphate buffered saline may be used. You will do either the large or small volume dilution procedure. Please ask your instructor which dilution procedure you will use.

Procedure: The tricky part of doing serial dilutions is determining the correct dilution to get 30-300 CFU's per plate. If you start out with a broth culture and do a 10-6 dilution, you should be in the right ballpark.

Large Volume Serial Dilutions:

Materials:

- 3 - 99mL sterile water bottles
- 100µL micropipettor with sterile tips
- 3 - 1mL sterile pipettes with blue pipetting aid
- 4 TSA plates (or other plate appropriate for your organism)
- 1 broth culture of organism

Diluting:

1. Label your three bottles 1:100, 1:10,000 and 1:1,000,000. In scientific notation this would be 10-2, 10-4, and 10-6.
2. Using a sterile 1mL pipette, transfer 1mL of your broth culture into the 10-2 bottle. Each bottle contains 99mL of sterile water.
3. Tightly cap the bottle, grab it in your hand, rest your elbow on the table and rapidly move your arm with the bottle in an arc, up and down, 25 times. This should adequately disperse the bacteria evenly throughout the bottle and break up bacterial clumps. This first bottle now has a 1:100 dilution of your original broth culture. There are still way too many bacteria in here to count if you were to plate them, so further dilution is necessary.
4. Using a new sterile 1mL pipette, transfer 1mL out of the first bottle (1:100 or 10-2) and add this to your bottle labeled 1:10,000 (10-4). Repeat the bottle shaking procedure. There are probably still too many bacteria in this dilution for you to successfully count, so the dilution process needs to be repeated once more.

Plating: Since you do not know which of your dilutions will yield countable results, you will plate from two of the three bottles.

1. Label two plates "0.1mL of 10-4" and "1mL of 10-4".
2. Label the other two plates "0.1mL of 10-6" and "1mL of 10-6".
3. Quickly shake the dilution bottle and aliquot the indicated amount from the appropriate tube onto the center of the plate. You can use either a 1mL pipette or your 100µL micropipetter, depending on the volume you are plating. Disperse it in 2-3 drops around the center of the plate.
4. Working quickly, use a sterile blue L-shaped spreader (looks like a little hockey stick) to spread the inoculum evenly around the plate. This is easier to do with the smaller inoculum volume. Do not invert the plates until all the liquid has absorbed into the surface of the agar.
5. Incubate the plates for the appropriate time and temperature. This is usually 24 hours at 35-37° Celsius.

8.3 Minimum Inhibitory Concentration

In a nutshell, to do an MIC, one inoculates the test substance with an invisible number of microorganisms and then observes the mixture of germs and test substance to see if it changes from clear to cloudy. If it turns cloudy, that means microorganisms have grown to high levels and the test substance is not inhibitory to them at that particular dilution. Test wells that remain clear after incubation may contain the original low-level inoculums of viable microorganisms, or the microbes could all have been killed by the antimicrobial agent. Those two outcomes cannot be differentiated visually. For that reason, scientists use MIC assays as indicators of an antimicrobial agent's inhibitory activity rather than biocidal activity.

Strengths of the Minimum Inhibitory Concentration Test (MIC Test):

The MIC test is relatively straightforward and easy to prepare for and execute, which naturally enhances reproducibility. The MIC test can be done on a very small scale without using too much antimicrobial agent. This is important for experimental antimicrobials such as biologically synthesized antimicrobial peptides. The MIC test is an easy way to test the antimicrobial attributes of a formulation across many different parameters, such as across microbial species or surfactant blends. Because little preparation is required for the minimum inhibitory concentration testing, test turnaround times can be kept low. Determination of MIC by microtiter plate method. The comparative minimum inhibitory concentration (MIC) of bacteriocin in supernatants and partial purified ones was determined by monitoring growth of an indicator strain in a 96 well microtiter plate.26 L. inoculate was applied as an indicator microorganism and multi-antibiotic resistant enterococci were subjected to evaluate the bacteriocin activity. In the first step, 100 μl broth media was added to each well in the microtiter plate then, 100 μl of a fraction to be tested for antimicrobial activity was added to the wells of column 1. In the next step, two-fold dilution of the sample was done from column 1 to column 2 and then continuing down 7 columns (the 8th was as a control column). Subsequently, overnight culture of indicator strain and resistant enterococci were diluted in fresh broth media to 1/20, prior to adding of 100 μl to each well. Finally, the microtiter

plate was incubated under conditions appropriate for the relevant indicator, and amount of growth was measured by reading optical density at 600 nm in a microtiter plate reader.[25]

RESULTS AND CONCLUSION

Quantitative analysis:-

S.No	Phytochemicals	Quantity(mg/100g)
1.	Total Phenols	0.1383
2.	Total Flavonoids	0.3383
3.	Tannins	0.077

Qualitative analysis

S.No	Phytochemicals	Result
1.	Tannins	Present
2.	Flavanoids	Present
3.	Saponins	Present

Antioxidant activity

% Inhibition of Ascorbic acid	% Inhibition of *Urtica diocia*
50.56%	3.2%
60.74%	15.31%
71.08%	18.9%
74.1%	24.3%
77.8%	34%
85.9%	44.4%

Antimicrobial activity:-

Urtica dioca and Pinus roxburghii hydroalcoholic extracts showed positive antimicrobial activity against S.aureus, E.coli, B.subtilis by performing above mentioned method.

These plants were showed more activity against S.aureus and B.subtilis than E.coli.

Hydrogels evaluation parameter:-

Degradation Rate:- The degradation of hydrogel was carried out for 1 week. No significant degradation occurred. Hence, degradation rate of the formulated hydrogel was low.

Swelling %:- The swelling % came out to be 53%.

CONCLUSION

In this study, we report the synthesis of polyacrylamide\chitosan hydrogel loaded with Urtica diocia having antimicrobial, antioxidant activity for wound healing using Qualitative, quantitative estimation, antioxidant and antimicrobial activity of plant extract. Antioxidant, antimicrobial activity of extract loaded hydrogels. Physicochemical properties of hydrogels like swelling index, degradation rate.

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