Accepted Manuscript

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PII: S0141-8130(17)30713-4
DOI: https://doi.org/10.1016/j.ijbiomac.2017.10.056
Reference: BIOMAC 8351

To appear in: *International Journal of Biological Macromolecules*

Received date: 25-2-2017
Revised date: 17-6-2017
Accepted date: 10-10-2017

Please cite this article as: Satish Patel, Shikha Srivastava, Manju Rawat Singh, Deependra Singh, Preparation and optimization of chitosan-gelatin films for sustained delivery of lupeol for wound healing, International Journal of Biological Macromolecules https://doi.org/10.1016/j.ijbiomac.2017.10.056

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Preparation and optimization of chitosan-gelatin films for sustained delivery of lupeol for wound healing

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Abstract

Lupeol entrapped chitosan-gelatin hydrogel (LCGH) films were prepared by solution cast method by blending chitosan and gelatin solution using glycerol as plasticizer, followed by crosslinking with glutaraldehyde. LCGH films were characterized by scanning electron microscopy (SEM), Fourier transform infrared spectroscopy (FTIR), differential scanning calorimetry (DSC), equilibrium water content (EWC), Water vapor transmission rate (WVTR) and *in vitro* release studies. SEM confirmed presence of the uniform porous network of both blank and LCGH films. The incorporation of lupeol in hydrogel was confirmed FTIR and DSC. The LCGH film was smooth, flexible, non-brittle and showed excellent swelling ability. EWC (85.40%) and WVTR (2228 ± 31.8) met the condition of ideal wound dressing. The biological activity of lupeol was assessed by antioxidant and antibacterial assay. Antioxidant assay confirmed that lupeol and LCGH film have excellent antioxidant properties by scavenging both radicals at steady increasing rate which increases with time due to steady release of lupeol. Antibacterial activity of lupeol in LCGH film was found to be retained as assessed by disc diffusion method. Cell viability was

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evaluated by MTT assay with NIH/3T3 fibroblast cells. The MTT assay showed that the CGH film evidently offered acceptable cell viability and non-toxicity. These observations depicted that chitosan/gelatin hydrogel film can be an ideal delivery system for sustained released of lupeol and LCGH film for enhanced wound healing.

Keywords: Chitosan; Hydrogel; Lupeol; Gelatin.

1. INTRODUCTION:

Varieties of dressings have been developed as drug delivery systems for management of wounds in the past. Over the last two decades, progressions in the understanding of pathophysiology of wounds has led to substantial biomedical innovations in the treatment of wounds including plant derived bioactives, drugs, growth factor, skin substitutes, and scaffolds. It has been suggested that wound healing is a complex series of cellular events that rebuild and restore the integrity and function of damaged skin **[1]**. Inflammatory phase is key phase in wound healing process. Various pro-inflammatory cytokines, growth factor and cells initiate proliferation and migration of new cells. Free radicals and reactive oxygen species released in this phase in presence of microbes at the wound site causes severe complications including infection, delayed healing process and severe wound dehydration. Dehydration interrupts ideal moist healing environment and further delay wound healing **[2]**.

Major limitation with traditional wound dressing and formulation as cream, gauze, cotton wool, gels etc are short residence time, leakage, poor patient compliance, least preservation of moist environment **[3]**. Modern wound dressing like hydrogels, hydrogel film provides moist environment, prevent tissue dehydration and cell death, enhanced migration of inflammatory cells and growth factor. It also allow gaseous exchange, enhanced angiogenesis, act as a barrier to microorganisms, remove excess exudates, have excellent biocompatibility and promote a rapid healing of wound **[4]**. These characteristics made hydrogel film close to ideal wound dressing.

Hydrogels are three-dimensional polymer complexes prepared by natural or synthetic polymer (hydrophilic) chains by physical or chemical crosslinking. It smartly responds to the various changes of temperature, ionic strength, pH of medium and presence of any enzyme. In the swollen form, they are soft and elastic, mimicking the tissue **[5, 6]**. Hence these are widely used in different field of pharmaceutical and biomedical engineering, drug delivery devices, artificial skin, and wound dressing production **[7]**.

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Chitosan and gelatin are the primary components used in preparation of wound dressing with the film forming nature **[8]**. Chitosan has been extensively used in wound dressing due to its hemostatic properties, biodegradability, biocompatibility, non-antigenic, non-toxic, regenerative effect on connective tissue, promotes tissue granulation and accelerates wound healing through the recruitment of inflammatory cells such as polymorphonuclear leukocytes and macrophages to the wound site **[9, 5]**. It is positively charged bio adhesive and forms gel easily. Gelatin is proteinaceous hydrocolloid biopolymer showing excellent properties like biodegradable, biocompatible, swellability to absorb vast water and easiness of chemical modification **[10]**. Numerous studies have proven that the films prepared using chitosan and gelatin combination have better results than when used alone in biomedical application. Mostly casting method is used for preparation of these films using acetic acid, calcium solvents systems and chitin/chitosan hydrogel **[11, 12, 13]**. In present study, lupeol loaded chitosan-gelatin film were prepared in presence of glutaraldehyde to enhance their common properties like biocompatibility and biodegradability **[14]**. Present study describes its surface morphology, crosslinking, thermal stability, equilibrium water content, water vapor transmission rate, *in vitro* release studies, and cell viability studies. Chitosan-gelatin combination has been shown to stimulate the adhesion, migration and growth of cells. **[15]** Both chitosan and gelatin in general exhibit healing properties with chitosan in particular exhibit antimicrobial and gelatin exhibit cell adhesion properties. Thus, it is anticipated that this unique combination as blend has immense potential to serve as a promising film forming matrix for wound healing.

Lupeol, a pentacyclic triterpene exhibit wound healing properties acting principally on inflammatory reaction and free radicals by regulating TNF-α, IL-2, IL-β specific mRNA, reduces PGE-2 synthesis from macrophage, reduces neutrophil migration, decrease myeloperoxide release, reduce IL-4 synthesis by Th-2 cells **[16].** Lupeol enhance the formation of extracellular matrix in proliferation phase by regulating collagen I through HSP47 expression modulation in human fibroblasts, prevent collagen I depletion and restore levels of hydroxyproline, hexosamine, hexuronic acid and matrix glycosaminoglycans **[17].** Collectively, considering these potential of lupeol, chitosan and gelatin, it was hypothesized that sustained delivery of lupeol at the wound site during healing will limit the infection, decreases the oxidative stress, continue the inflammatory phase, enhance tissue regeneration and remodeling of extra cellular matrix by action of these bioactives on different phases of wound healing. In present study, the lupeol loaded hydrogel film

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was prepared from the blended mixture of chitosan and gelatin using glutaraldehyde as linker and characterized by various parameters.

2. EXPERIMENTAL

2.1 Materials

Lupeol was purchased from Sigma (India) and chitosan (medium molecular weight, decetylation between 75-85%), gelatin and glutaraldehyde from Himedia Laboratories (India). All other chemicals used in the studies were of analytical grade. Ultrapure water from Milli-Q water system was used to prepare the aqueous and buffer solutions.

2.2 Preparation of Chitosan/Gelatin hydrogel film

Chitosan solution was prepared by adding 2g chitosan in 100 ml of 1% acetic acid and the solution was stirred for 2 h, Glycerol (0.3 ml) was added in this solution as plasticizer. 2.5% w/v gelatin solution was prepared by dissolving the gelatin powder in distilled water under stirring for 30 min. Glycerol (0.7 ml) was added in this solution as plasticizer. Chitosan solution was then mixed with the gelatin solution and stirred for 12 h at 40ºC in the different ratios **(Table 1)**. 3 ml of 0.25% glutaraldehyde solution was added into each mixture to crosslink chitosan and gelatin and stirred for 6 h at room temperature. Lupeol was dissolved in dimethylsulfoxide and added to the mixtures with stirring at room temperature for homogenization. Hydrogel film was prepared by solution casting method by pouring solution on a petridish at a thickness of 2-5 mm, followed by standing and cooling at 35ºC to form film. Prepared film was kept under ultraviolet rays' for sterilization for 30 minutes **[18, 10].** Prepared films were carefully removed from petridish, wrapped in parafilm and kept in desiccator.

2.2.1 Determination of free glutaraldehyde

Chromotropic acid (CTA) reagent was used for determination of excess aldehyde/formaldehyde content. CTA reagent was prepared by dissolving 50mg of sodium salt of chromotropic acid in 100 ml of concentrated hydrochloric acid **[19, 20]**. In present study, CTA reagent was used for estimation of glutaraldehyde in chitosan-gelatin solution after crosslinking with glutaraldehyde (CGG solution). CGG solution (5 ml) was transferred to 25ml glass stoppered test tube and 10 ml of CTA reagent was added. The control solution was prepared by adding 3 ml of 0.25% glutaraldehyde solution and 10 ml of CTA reagent. The reaction mixture was kept in water bath

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for 30 minute and absorbance (light yellow color change to purple color) was measured at 580 nm in UV/Vis spectrophotometer (Schimadzu UV 1800, Japan). The test was done in triplicate.

2.3 Hydrogel film Characterization

2.3.1 Physical characteristics

LCGH films were characterized in terms of weight uniformity. Each, five specimens of size 2.0 cm x 2.0 cm of all films were weighed on electronic balance (Shimadzu, Japan) and mean weight was calculated. The thickness of LCGH films were measured by using an electronic micrometer (U-Therm, China) at five different positions of the film with 0.001 mm of accuracy. The result was expressed as a mean of the measurements ± standard deviation (SD).

2.3.2 Morphology

The surface morphology of the CGH film and LCGH film (F-3, R = 0.5) were investigated by scanning electron microscopy (Carl Zeiss AG - EVO40, Germany). The samples were freeze-dried, then mounted on the standard specimen mounting stubs and were coated with a thin layer (20nm) of gold by sputter coater unit.

2.3.3 FTIR Spectral analysis

The FTIR spectral analysis was used to assess the chemical composition of the drug and polymers in raw form, and to evaluate the probable interactions between the compounds in prepared film. The analysis of the prepared hydrogels were performed on FTIR spectrophotometer (Shimadzu 8400S, Japan) in a range of 4000-500 cm^{-1} using a 500 mg potassium bromide pellet having 5mg of the sample.

2.3.4 Differential calorimetry scanning

Differential scanning calorimetry (DSC) studies of lupeol, chitosan, gelatin and the lyophilized lupeol loaded hydrogel powder were carried out to evaluate the thermal stability of hydrogel. Accurately weighed samples were carefully placed in DSC sample holder and calorimetric curves were recorded in range of 25–350˚C at a heating rate of 10˚C/min under a nitrogen environment (50.0 ml/min) from DSC Q10 V9.4 Build 287 (Shimadzu DSC-60 Systems, Japan).

2.3.5 Water vapor transmission rate (WVTR)

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The moisture permeability of the LCGH films was evaluated by determining the water vapor transmission rate across the films. The WVTR test was performed using the upright wet cup method according to JIS 1099A standard **[21]**. A circular piece of the specimen was affixed over the top of a permeability cup of 6 cm in diameter having 50 g of $CaCl_2$ using water-resistant tape to avoid any moisture loss through the edges. The cup was then placed in an incubator at 90±5% RH at 40±2ºC. The WVTR was calculated by following formula:

$$WVTR = \frac{W2-W1}{S} X\ 100 \frac{g}{m2}/day$$

W_1 and W_2 are the weights of permeability cup at the end of 1st and 2nd hour respectively and S is the area of the hydrogel films.

2.3.6 Equilibrium water content (EWC) study:

The equilibrium swelling behavior of LCGH films in deionized water was studied to know about their equilibrium water content or moisture content in room temperature. The LCGH films were dipped into deionized water for 24 hours and the wet weights were determined after first blotting with filter paper followed by blowing with a steam air to remove the surface water and weighing the swollen hydrogel **[22]**. The EWC was calculated from the following equation:

$$EWC\ (\%) = \frac{We - Wd}{We} X\ 100$$

Where, W_e and W_d are the weight of the swollen hydrogel at equilibrium and the dried hydrogel films respectively.

2.3.7 Drug release study:

An *in vitro* release study of film (F-3) was carried out in dissolution apparatus (Electrolab, India) **[23]**. Phosphate buffer with pH 7.4 was used as diffusion medium. The LCGH film (F-3) was placed in dissolution apparatus containing buffer of pH 7.4 and speed was fixed 50 rpm±5 at 37°C±0.5. Samples were withdrawn each from the apparatus using 1 ml pipette at regular time internal (0–6, 8, 10 and 12 h). The volumes of withdrawn samples were replaced with buffer (pH 7.4) to maintain sink condition. The absorbance of the samples, after filtration, was measured with a double beam spectrophotometer (Shimadzu UV 1800, Japan) at 350 nm. From the value of absorbance, % drug release was calculated and graph of % drug release against time was plotted. Release studies were performed in triplicate.

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2.4 Antioxidant activity

2.4.1 DPPH free radical scavenging activity

The antioxidant activity of the lupeol and LCGH film was assayed spectrophotometrically as per the protocol described by Nallamuthu with slight modification **[24]**. Briefly, 1 ml of 0.004 % DPPH solution in ethanol was mixed with 1 ml of different lupeol (5, 25, 50, 500 µg/ml) concentrations. LCGH film (1X1 cm) was added to 5ml of ethanol and 5ml of 0.004% DPPH solution followed by agitation of mixture. Control solution was prepared by adding 1ml of DPPH solution (0.004%) in 1 ml of ethanol. The reaction mixtures were kept at room temperature for 45 minute and absorbance (color change from violet to light yellow) was measured at 519 nm in the UV/Vis spectrophotometer (Schimadzu UV 1800, Japan). Absorbance of reaction mixture of LCGH film was measured at regular time interval (1-6 h) **(Table 2)**. The assay was done in triplicate for each reaction mixture. The decrease in absorbance of DPPH and on the addition of lupeol and LCGH film in comparison to the control was calculated as:

$$Scavenging\ activity\ (\%) = \left[\frac{Absorbance\ of\ Control -\ Absorbance\ of\ sample}{Absorbance\ of\ contol}\right] X\ 100$$

2.4.2 Nitro-blue tetrazolium (NBT) assay

In-vitro super oxide radical scavenging activity was performed by preparing reaction mixture containing 0.2ml of Riboflavin solution (1 mg/ml solution in Methanol), 0.2ml of NBT (1 mg/ml solution in Methanol) and 0.4 ml EDTA solution (12mM) were mixed and phosphate buffer (pH 7.4) was added to make volume of 10 ml **[25]**. This reaction mixture was taken as control. Briefly, 0.4 mL of varying concentration (5, 25, 50, 500 µg/mL) of lupeol was mixed with control mixture. The LCGH film (1X1 cm) was added to 5 ml of phosphate buffer and 5ml of control reaction mixture and agitated. Samples were withdrawn from this at regular time interval (1-6 h) and mixed with control reaction mixture. Absorbance (color change from light yellow to blue) was measured at 560 nm after exposing each sample to light for 15 minutes **[26]**. The assay was done in triplicate for each reaction mixture. The decrease in absorbance of samples was calculated as in DPPH assay.

2.4.3 Antibacterial assay:

The Lupeol released from swollen LCGH films were tested for antibacterial activity. This was done by the disc diffusion method using *P. aeruginosa* (MTCC 424) as test organism kept in

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nutrient media **[27, 28, 29]**. Different dilutions (5-25µg/ml) of pure lupeol at different concentrations were assessed for inhibition activity and calibration curve was prepared. The antibacterial activity of LCGH films were evaluated by various samples of lupeol release at different time intervals (0.5–6, 8, 10 and 12 h). Nutrient agar media was used to prepare agar plate by pouring into sterilized petridish and solidified. The prepared culture plates were inoculated with the microbe culture and marked. Wells were made in plates using a sterilized well borer (6 mm holes, 25 mm distance from each other). Eleven wells were made for dilution of 0, 5, 10, 15, 20, 25 µg/ml of pure lupeol and dilution of samples from 1, 6, 8, 10 and 12 h The sample (10 ml) collected (in vitro drug release aliquots and pure lupeol solutions) were filtered through sterilized membrane filters (0.45 µm) and cautiously filled into the wells. These samples (50µl) in wells were kept to diffuse for 2 h. After diffusion of sample in agar plates, incubated for 18 h at 37±0.5°C. By using a caliper, the diameter of inhibition zone surrounding well was observed. Lupeol concentration from inhibition diameters was compared with the concentration from release method. Each experiment was carried out in triplicate.

2.4.4 Cell viability assay

The cytotoxicity/ biocompatibility of the prepared CGH film was evaluated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. This method is based exposing any viable cell to test sample in presence of MTT. The solutions of CGH film were diluted with media (0%, 12.5%, 25%, 50%, and 100%) **[30]**. Medium without any CGH film sample i.e. 100% media was used as control. These dilutions were incubated with Dulbecco's Modified Eagles Medium (DMEM) media at 37ºC for 1h in an incubator. These dilution were added to a 96-well plates containing 3×10^5 cells/ml NIH/3T3 fibroblast cells (200 µl) in DMEM media incubated at 37ºC for 24h in a 5% CO_2 humidified atmosphere. After this, DMEM media was discarded and cells were rinsed with phosphate buffered saline. Then, 5 mg/ml MTT solution (10 µl) was added to each well and incubated for 4 h at 37ºC. Hereafter, the medium was removed completely from each well, and then 200 µl of 40 mM DMSO lysis buffer was added to each well. One hundred µl of cell lysate was transferred into each well of a 96-well plate. Absorbance at 570 nm was measured with a microplate reader (SpecraMax M2e; Bucher Biotech, Basel, Switzerland) to determine the cell viability. Data are shown as values relative to the control with a control value of 100 percent. Each experiment was carried out in triplicate.

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$$Cell\ viability = \frac{Absorbance\ of\ sample}{Absorbance\ of\ control} X100$$

3. Result and Discussion

3.1 Formulation, optimization, and physical characteristics of hydrogel film

Hydrogel film prepared from chitosan alone was too brittle in nature and tough to remove from petridish. Therefore blend mixture of chitosan and gelatin in different ratios were used. Chitosan and gelatin have proven wound healing properties individually. So their combination is also expected to show improved response in wound management. In these mixtures, formulation F-3 was very thin, uniform, flexible, non-brittle and easy to remove from petridish as compared to F-1 and F-2. Addition of glycerol increased its flexibility and thickness due to interpenetration into polymer network **[31]**. The weight of each prepared hydrogel films (2cm X 2cm) were found to range from 0.26g to 0.380g **(Table 1)**. The thickness of prepared LCGH films ranged between 0.452-0.719mm **(Table 1)**. Two mechanism may involve in this crosslinking reaction between chitosan, gelatin and glutaraldehyde i.e. Schiff-base mechanism and acetalization reaction **[32]**. Both mechanisms were responsible for polymerization/crosslinking of chitosan with glutaraldehyde while Schiff-base mechanism was involved in crosslinking of gelatin with glutaraldehyde. Schiff base formation of –C=N (imine bond) occurred between free amino group of gelatin and chitosan and aldehyde group of glutaraldehyde. The bond may be formed by non-uniform chains or terminal unities **[33]**. Acetalization reaction occurred between hydroxyl groups (–OH) in chitosan and aldehyde group in glutaraldehyde lead to formation of C-O-C-O-C bond. Possible chemical reaction has been shown in **Fig. 1.**

3.3.1 Determination of free glutaraldehyde

Crosslinking of amino group is increased by aldehyde group viz. glutaraldehyde, formaldehyde, glyceraldehyde etc. Aldehyde group crosslinks with amine group and forms imine bonds. This concept used for determination of crosslinking of untreated/free glutaraldehyde in reaction mixture. Aldehyde group condense with chromotropic acid in presence of hydrochloric acid and converted to p-quinoidal form (Purple color) and show maximum absorbance at 580 nm **[19, 20]**. In present study, results showed that color (light yellow) of CGG solution was not changed to purple as compare to control solution. Absorbances at 580 nm are shown in **Table 1 in**

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supplementary data. This assay confirmed that all the glutaraldehyde was used in crosslinking of chitosan/gelatin and glutaraldehyde.

3.2 Morphology

The SEM images of the CGH film and LCGH film (F-3) are shown in **Fig. 2**. Surface morphology of both films was relatively smooth, fibrous and porous in nature. The large pores were not observed on the surface and edges of films. This smooth morphology was due to the presence of chitosan in the hydrogel films. Photomicrographs showed that the gelatin and chitosan hydrogel were linked at molecular level. **Fig 2b** clearly shows that the drug incorporated in the hydrogel did not affect the structure of the hydrogel. This porous and fibrous nature film will aid in wound healing by increasing the oxygen supply to wound **[34]**.

3.3 FTIR analysis

FT-IR spectra analyses were performed to confirm the chemical nature of hydrogel and crosslinking between chitosan and gelatin. **Fig 3** shows the FT-IR spectrum of chitosan, gelatin, and chitosan-gelatin hydrogel film. Chitosan has broad peak at 3400.53 cm^{-1} indicating stretching vibration due to N-H and O-H bond **[35]**. Stretching vibration at 2849.78 cm^{-1} and bending vibration at 1400.53 cm^{-1} show the presence of methylene group in CH_2OH and methyne group in pyranose ring. Absorption in range of 1070.68cm^{-1} -1111.56cm^{-1} was due to asymmetric vibrations of CO **[36]**. Peak at 1592.23cm^{-1} confirmed the deacetylation and indicate occurrence of NH_2 group **[37]**. In spectra of pure gelatin, broad peak at 3215.38cm^{-1} indicate N-H stretching and 1592.38 indicate C=O stretching. Peak at 1400.31cm^{-1} and 1339.55cm^{-1} was due to the presence of amide bond **[38]**. In spectrum of hydrogel, peak having increased frequency at 3349.81cm^{-1} indicate the interaction between C=O group of gelatin with N-H and O-H group of chitosan and this peak shifting might be due to Schiff base formation between free amino group of gelatin and aldehyde group of glutaraldehyde. The reduction of hydroxyl absorbance also showed that CGH film was prepared successfully by crosslinking. The hydroxyls groups of polymer had been consumed during crosslinking reaction with glutaraldehyde under acidic condition and acetal groups. Peak at 1680 cm^{-1} showed strong absorption due to acetal formation by glutaraldehyde crosslinked chitosan. Peak at 1645 cm^{-1} might be due to cross-linked gelatin **[32, 39].** In spectrum of CGH film did not indicate any presence of unreacted glutaraldehyde. This interaction causes increased N-H

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bending and decreased C-O stretching vibration in both chitosan and gelatin at 1073cm^{-1} and 1111cm^{-1} respectively **[40]**.

3.4 Differential calorimetry scanning

DSC has been recently used for determination of thermal stability of biomaterials**. Fig 4** shows the DSC thermograms of Lupeol, Chitosan, Gelatin, and hydrogel. The DSC thermogram of lupeol showed glass transition temperature (Tg) 175.31˚C and a sharp endothermic peak at 215.39˚C corresponding to its melting point and characteristic crystalline nature (**Fig 4a**). Tg of Chitosan and gelatin were 164.52˚C and 140.38˚C respectively and both show a sharp endothermic event, assigning to the melting endotherm at 114.28˚C **(Fig 4b)** and 96.01˚C **(Fig 4c)** respectively. DSC thermogram of drug in LCGH film had shown Tg at 201.97˚C and 132.08˚C and melting endotherm at 186.01˚C, and 80.25˚C. Shifting of peaks in comparison of pure drug and polymer and appearance of some new peaks indicated the presence of interaction between polymers and incorporation of drug (**Fig 4d**). The process for all tested ingredients was endothermic. This shows that heat absorption occur but without dropping mass. This peak shows an intermediate energy utilized for the combination of chitosan and gelatin with lupeol when compared with pure drug and polymer **[41]**. In LCGH film thermogram, Tg at 29.88˚C indicate that storage temperature for preparation should not exceed 30˚C as structural changes might occur after long time storage.

3.5 Water vapor transmission rate (WVTR)

For natural healing process, wound dressing must control the moisture loss from wound. The WVTR for normal skin is 204g/m^2/day and for wounded skin from 279 to 5138 g/m^2/day depending on the type and nature of the wound. Water vapor transmittance rate of 2000-2500 g/m^2 /day would provide a suitable level of moisture to avoid excessive dehydration and proper wound healing **[42]**. Higher WVTR increase the formation of scar by drying the wound area quickly while low WVTR increases the chance of bacterial infection by accumulating exudates and delayed healing process **[21]**. The effect of chitosan and gelatin on the WVTR of the hydrogel is given in Fig. 1a in supplementary data. WVTR increased as amount of chitosan increased relative to gelatin from 1545 ± 3.6 g/m^2/day to 2228 ±31.8 g/m^2/day. The WVTR of hydrogel film (F-3) was 2228 ±31.8 g/m^2/day, close to WVTR of ideal wound dressing. Thus selected hydrogel film (F-3) maintains natural moisture environment for wound healing.

3.5 Equilibrium water content (EWC) study:

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Fig 1b in supplementary data and Table 1 depicted that the chitosan content increases, % EWC is also increased. Formulation F-3 showed highest % EWC (**Table 2 supplementary data**). Equilibrium water content of any film is directly connected with its porosity and hydrophilic group present in the film. Both chitosan and gelatin having hydrophilic groups like alcoholic (-OH) and amine (-NH2) and having swelling properties to absorb vast water content **[9, 10]**. Water absorption capacity increases due to pores network formed in hydrogel film. Lower value of % EWC of film F-1 and F-2 may be due to its thickness.

3.6 Drug release study:

In vitro release studies were carried out for film (F-3) using dissolution apparatus over a period of 12 h. Results displayed that release of lupeol from F-3 followed a biphasic release pattern with initial burst release followed by constant release of lupeol with about 90.99 ± 1.27% after 24 h (Fig. 2 in supplementary data). This might be due to the diffusion of drug from the surface of film in the first hour followed by diffusion from the entrapped drug. Initial burst release and subsequent sustained release are crucial desirable parameters. Burst release is beneficial to improve the penetration of drug and sustained release for a prolonged period of time **[23]**. Initially released lupeol help in recruitment of proinflammatory cytokine and limit infection. Further constant release of lupeol promote the wound healing phase by its anti-inflammatory, free radical scavenging free radicals and superoxide ions suppressive properties and increases the collagen and fibronectin synthesis **[43, 16]**. Lupeol released from hydrogel formulation followed first order release kinetics as compared to different kinetic models and their linear regression was measured. The kinetic parameter values (R^2 and constants) of five kinetic models of lupeol release data are shown in supplementary data (**Table 3 supplementary data**). The obtained values showed that Higuchil and Hixon Crowell models also show good linearity after first order model. Sustained and constant drug release was observed for lupeol during the study. In controlled or sustained release formulations, three mechanisms that control the release are diffusion, swelling and erosion. The lupeol release from the prepared nanoparticle is mainly by the diffusion and described by Fickian diffusion. But in case of hydrogel, swelling and diffusion play an important role in release mechanisms. So, release profiles from formulation studied are described by Fick's second law of diffusion **[44]**.

3.7 Antioxidant activity:

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Accumulation of free radicals and reactive oxygen species at wound site delay healing process due to their harmful effects on inflammatory cells and tissues **[26]**. Lupeol play a crucial role in decreasing free radicals and promoting wound-healing process **[45]**. DPPH and NBT assay confirmed the antioxidant potential of formulation. The DPPH free radical-scavenging effects of lupeol at different concentration and lupeol loaded hydrogel film (F-3) at different time interval are shown in Table 2 and Fig. 3 in supplementary data. Results have shown that as lupeol was added to reaction mixture, purple color of DPPH changed to light yellow color (Absorbance decreases) confirming free radical scavenging action. The principal of assay is the reduction of DPPH. DPPH solution (purple color) gives strong absorbance at 519 nm due to presence of odd electrons. Addition of lupeol decreases the intensity of absorption and induces color change due to increase in hydrogen donor (-OH) concentration with more electrons captured **[46]**. The amount of colour change is directly proportional to antioxidant activity of sample. Decrease in the absorbance of the reaction mixture is directly correlated to significant free radical scavenging activity of sample.

Super oxide radical scavenging assay was performed using NBT, riboflavin and sample. The Super oxide radical scavenging effects of lupeol at different concentration and lupeol loaded hydrogel film (F-3) at different time interval are shown in Table 2 and Fig. 4 in supplementary data. Super oxide radicals were produced from light induced oxidation of riboflavin. These radicals reduced the NBT to blue colored formazan when measured at 560 nm. Lupeol in the sample prevented their interaction with NBT and decreased the absorbance and color formation. This may be due to electron donating group –OH and $–CH_3$ **[47]**.

In both assay, lupeol showed potent antioxidant activity which further increased as concentration of lupeol increased. Prepared formulation of hydrogel film (F-3) showed increase in activity with time might be due to steady release of lupeol. Thus, lupeol formulation can protect injured tissues from oxidative damage and significantly improve healing process.

3.8 Antibacterial assay:

Antibacterial activity of lupeol was observed using disc diffusion method. Lupeol released from LCGH films showed inhibition zone in prepared agar culture plates. Inhibition zone was observed in each samples bore. The antibacterial activity of lupeol was calculated by measuring zone of inhibition. The diameter of zone of inhibition increased with each sample from first hour to 12

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hour [Table 3 and Fig. 5 (supplementary data)]. This might be attributed to constant release of lupeol with time leading to increase in the inhibition zone on agar plates. This assay also confirms that antibacterial activity of lupeol does not get affected by entrapment procedures.

3.9 Cell viability assay

The effect of prepared CGH film on cell viability (cytotoxicity) was examined using the MTT assay, which is a colorimetric method to determine the metabolic activity of viable cells. The result of MTT assay showed satisfactory cell viability on CGH film dilutions.

All the dilution of CGH film extracts had shown no toxicity as more than 90% of cells were viable and proliferating **(Fig 5)**. The results clearly showed that CGH film have capacity to stimulate the cell viability to 116.71% which is 35% higher than that of the control value. Figure 4a shows the Box & Whisker plot of the % cell viability of NIH/3T3 fibroblast cells. The box signifies the point of 50% of the values and the line in the box displays the median values **[48].** The MTT assay is based on the conversion of insoluble purple formazon crystals from soluble yellow tetrazolinum salt by enzyme mitochondrial dehydrogenase of living cells that is measured in a spectrophotometer **[30, 49]**. The absorbance corresponds to the number of living cells. Thus CGH film evidently offered acceptable cell viability and non-toxicity.

Conclusion

From these observations, it is concluded that hydrogel film produced from chitosan-gelatin by solution cast method using glyceraldehyde as cross-linker showed uniform surface morphology with non-brittle, fibrous and porous nature. Film offers flexibility by addition of glycerol and meets all the specifications of an ideal wound dressing. FTIR spectrum and DSC thermogram confirm the incorporation of lupeol in hydrogel system. Film (F-3) plasticized by glycerol significantly changed the hydrogel properties, strength, thickness, and showed high swelling capacity and controlled release up to 24 h. The film provides natural environment due to simulated WVTR equilibrium water content along with excellent antioxidant and antibacterial properties of polymer and lupeol. Release studies confirmed that this polymer-based dressing can be an effective support for lupeol release into wound. The MTT assay showed that the CGH film evidently offered acceptable cell viability and non-toxicity. This formulation might have the potential to be used as a film dressing for drug delivery in wound management.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the article.

Acknowledgement

The authors are thankful to Director, University Institute of Pharmacy, Pt. Ravishankar Shukla University, Raipur (C.G.) India. This work is supported by UGC-MRP-41-748-2012, DHR-HRD/FELLOWSHIP/SUG-01 (15-16), UGC-BSR-7-341-2011, UGC-SAP Phase II for financial assistance.

References

1. D. Singh, M. Singh, S. Saraf, S. Saraf, Development of delivery cargoes for debriding enzymes effective in wound healing, Trend. Appl. Sci. Res. 6 (2011) 863-876.
2. Z. Fan, B. Liu, J. Wang, S. Zhang, Q. Lin, P. Gong, L. Ma, S. Yang, A novel wound dressing based on Ag/Graphene polymer hydrogel: effectively kill bacteria and accelerate wound healing, Adv. Funct. Mater. (2014) 1-11. DOI: 10.1002/adfm.201304202
3. J.S. Boateng, K.H. Matthews, H.N.E. Stevens, G. M. Eccleston, Wound healing dressings and drug delivery systems: A Review, J. Pharm. Sci. 97, 8 (2008) 2892-2923.
4. F.K. Field, M.D. Kerstein, Overview of wound healing in a moist environment, Am. J. Surg. 167 (1994) 2S-6S.
5. M.R. Singh, S. Patel S, Singh, Natural polymer-based hydrogels as scaffolds for tissue engineering. In Nanobiomaterials in Soft Tissue Engineering Applications of Nanobiomaterials edt by A. M. Grumezescu, Elsiver, (2016) 231-253. DOI: http://dx.doi.org/10.1016/B978-0-323-42865-1.00009-X
6. M.R Singh, S. Saraf, A. Vyas, V. Jain, D. Singh, Innovative approaches in wound healing: trajectory and advances, Art. Cell. Nanomed. Biotech. 41 (2013) 202-212.
7. M. Park, H. Y. Shin, B. S. Kim, M. J. Kim, B. Y. Park, H. Y. Kim, I. S. Kim, Effect of discarded keratin-based biocomposite hydrogels on the wound healing process in vivo. Mat. Sci. Eng. 55 (2015) 88–94.

ACCEPTED MANUSCRIPT

8. Z. Nasreen, M.A. Khan, A.I. Mustafa, Improved Biodegradable Radiation Cured Polymeric Film Prepared from Chitosan-Gelatin Blend, J. Appl. Chem. (2016) 11. http://dx.doi.org/10.1155/2016/5373670

9. L.I. Moura, A.M. Dias, E. Carvalho, H.C. de Sousa, Recent advances on the development of wound dressings for diabetic foot ulcer treatment-a review, Acta. Biomater. 9 (2013) 7093–7114

10. L. H. Peng, X. Chen, L. Chen, N. Li, W.Q. Liang, J. Q. Gao, Topical Astragaloside IV-Releasing Hydrogel Improves Healing of Skin Wounds in Vivo, Biol. Pharm. Bull. 35, 6 (2012) 881–888.

11. E. Shahrama, S. H. Sadraie, G. Kaka, H. Khoshmohabat, M. Hosseinalipour, F. Panahi, M.R. Naimi-Jamal, Evaluation of chitosanegelatin films for use as postoperative adhesion barrier in rat cecum model. Int. J. Sur. 11 (2013) 1097-1102.

12. H. Nagahama, H. Maeda, T. Kashiki, R. Jayakumar, T. Furuike, H. Tamura, Preparation and characterization of novel chitosan/gelatin membranes using chitosan hydrogel. Carbohyd. Pol. 76 (2009) 255–260.

13. C. Mingyu, D. Jinguang, F. Yang, G. Yandao, Z. Nanming, X. Zhang, Study on physical properties and nerve cell affinity of composite films from chitosan and gelatin solutions. Biomater. 24 (2003) 2871.

14. C.Y. Lii, P. Tomasik, H. Zaleska, S.C. Liaw, V.M.F. Lai, Carboxymethyl cellulose-gelatin complexes,Carbohyd. Pol. 50, 1 (2002)19-26.

15. Y. Liu, M. An, L. Wang, H, Qiu., Preparation and Characterization of Chitosan-Gelatin/Glutaraldehyde Scaffolds. J. Macromol. Sci., Part B: Phy. 53 (2014) 309-325, DOI: 10.1080/00222348.2013.837290

16. D.L. Lucetti, E.C.P. Lucetti, M.A.M. Bandeira, H.N.H. Veras, A.H. Silva, L.K.A.M. Leal, Anti-inflammatory effects and possible mechanism of action of lu peol acetate isolated from *Himatanthus drasticus* (Mart.) Plumel, J. Inflam., 7 (2010) 60. DOI: 10.1186/1476-9255-7-60

17. T. Geetha, P. Varalakshmi , Anti-inflammatory activity of lupeol and Lupeol linoleate in rats, J. Ethnopharmacol. 76 (2001) 77–80.

ACCEPTED MANUSCRIPT

18. T. Wang, X.K. Zhu, X.T. Xue, D.Y. Wu. Hydrogel sheets of chitosan, honey and gelatin as burn wound dressings, Carbohydr. Pol. 88 (2012) 75–83.

19. R.N. Kale, A.N. Bajaj. Ultraviolet spectrophotometric method for determination of gelatin crosslinking in the presence of amino groups. J. Young. Pharm. 2, 1, (2010) 90-94.

20. A. Dar, U. Shafique, J. Anwar, W. Zaman, A. Naseer. A simple spot test quantification method to determine formaldehyde in aqueous samples. J. Saudi. Chem. Soc. 20, (2016) s352-s356.

21. M.R. Hwang, J.O. Kim, J.H. Lee, Y.I. Kim, J.H. Kim, S.W. Chang, et al., Gentamicin-loaded wound dressing with polyvinyl alcohol/dextran hydrogel: gel characterization and in vivo healing evaluation. AAPS Pharm. Sci. Tech. 11, 3 (2010) 1092-1103.

22. H.J. Yoo, H.D. Kim, Synthesis and properties of waterborne polyurethane hydrogels for wound healing dressings, J. Biomed. Mater. Res. B Appl. Biomater. 85, 2, (2008) 326

23. M. Pradhan, D. Singh, M.R. Singh, Development characterization and skin permeating potential of lipid based novel delivery system for topical treatment of psoriasis. Chem. Phys. Lipids. 186 (2015) 9-16.

24. I. Nallamuthu, A. Parthasarathi, F. Khanum, Thymoquinone-loaded PLGA nanoparticles: antioxidant and anti-microbial properties. Int. Curr, Pharma. J. 2 (2013) 202-207.

25. R.M. Patel, N. Patel. *In vitro* antioxidant activity of coumarin compounds by DPPH, Super oxide and nitric oxide free radical scavenging methods. J. Adv. Pharm. Edu. Res. 1 (2011) 52-68.

26. M.E. Shahira, A. M.A. Choucry, Z.A. Kandil, Antibacterial, antioxidant, and topicalanti-inflammatory activities of *Bergia ammannioides*: A wound-healing plant, Pharm. Biol. (2015) 1-10. DOI: 10.3109/13880209.2015.1028079

27. D. Singh, M. Singh , S. Saraf , V.K. Dixit, S. Saraf , Characterization of gentamicin loaded chitosan microspheres for effective wound healing, Indian J. Pharm. Educ. Res. 44, 2 (2010) 171-182.

ACCEPTED MANUSCRIPT

28. K. Vimala, Y.M. Mohan, K. Varaprasad, N. Reddy, S. N. Ravindra, N.S. Naidu, et al., Fabrication of curcumin encapsulated chitosan–PVA silver nano composite films for improved antimicrobial activity. J. Biomat. Nanobiotech. 2 (2011) 55–64.
29. M. Balouiri, M. Sadiki, S.K. Ibnsouda, Methods for in vitro evaluating antimicrobial activity: A review. J. Pharma. Anal. 6 (2016) 71–79.
30. E.E. Hago, X. Li., Interpenetrating Polymer Network Hydrogels Based on Gelatin and PVA by Biocompatible Approaches: Synthesis and Characterization. Adv. Mat. Sci. Engineer.(2013) 8. http://dx.doi.org/10.1155/2013/328763
31. H.V. Pawar, J. Tetteh, J.S. Boateng, Preparation, optimization and characterization of novel wound healing film dressings loaded with streptomycin and diclofenac. Colloid. Sur. B: Biointer. 102 (2013) 102–110.
32. Y.F. Qian, K.H. Zhang, F. Chen, Q.F. Ke, X.M. Mo, Crosslinking of gelatin and chitosan complex nanofibers for tissue engineering scaffolds. J. Biomater. Sci. Polym. Ed. 22 (8) (2011) 1099-113.
33. O.A. Monteiro, C. Airoldi. Some studies of crosslinking chitosan-glutaraldehyde interaction in a homogenous system. Int. j. Biol. Macromol. 26, 2-3, (1999) 119-28.
34. N. Natarajan, V. Shashirekha, S.E. Noorjahan, M. Rameshkumar, C. Rose, T.P. Sastry, Fibrin–Chitosan–Gelatin Composite Film: Preparation and Characterization. J. Macromol. Sci., Part A, 42, 7 (2005) 945-953. DOI: 10.1081/MA-200063188
35. S. Kumari, P.K. Rath, Extraction and Characterization of Chitin and Chitosan from (*Labeo rohit*) Fish Scales. Proc. Mat. Sci. 6 (2014) 482–489.
36. S.M.L. Silva, R.C. Carla, C.R.C. Braga, M.V.L. Fook, C.M.O. Raposo, L.H. Carvalho, E.L. Canedo, Application of Infrared Spectroscopy to Analysis of Chitosan/Clay Nanocomposites, Infrared Spectroscopy - Materials Science, Engineering and Technology, Prof. Theophanides Theophile (Ed.), (2012) ISBN: 978-953-51-0537-4.
37. R.H. Marchessault, F. Ravenelle, X.X. Zhu, Polysaccharides for drug delivery and pharmaceutical applications. Washington, DC: American Chemical Society, (2006).
38. J. Kong, S. Yu, Fourier transform infrared spectroscopic analysis of protein secondary structures, Acta. Biochimica. et Biophysica. Sinica. 39 (2007) 549- 559.

ACCEPTED MANUSCRIPT

39. S. Distantina, Rochmadi, M. Fahrurrozi, Wiratni, Preparation and Characterization of Glutaraldehyde-Crosslinked Kappa Carrageenan Hydrogel. Eng. J. 17 (2013) 57-66.

40. S. Kim, E. Marcel, Z. Yang, B. Han, Chitosan/Gelatin–Based Films Crosslinked by Proanthocyanidin. J. Biomed. Mater. Res. B. Appl. Biomater. 75, 2 (2005) 442-50.

41. M.O.G. Ferreira, L.L.R. Leite, I.S. Lima, H.M. Bareto, H.M., L.C.C. Nunes, et al., 2016. Chitosan hydrogel in combination with nerolidol for healing wounds. Carb. Pol. 152 (2016) 409-418.

42. D. Queen, J.D.S. Gaylor, J.H. Evans, J.M. Courtney, W.H. Reid, The preclinical evaluation of the water vapour transmission rate through burn wound dressings. Biomater. 8 (1987) 367–71.

43. M. Na, B.Y. Kim, H. Osada, J.S. Ahn, Inhibition of protein tyrosine phosphatase 1B by lupeol and lupenone isolated from *Sorbus commixta*. J. Enzym. Inhib. Med. Chme. 24 (2009) 1056–9.

44. J. Siepmann, N.A. Peppas, Modeling of drug release from delivery systems based on hydroxypropyl methylcellulose (HPMC), Adv. Drug Deliv. Rev. 48 (2001) 139–157.

45. Tchimene MK, C.O. Nwaehujor, M. Ezenwali, C.C. Okoli, M.M. Iwu, Free Radical Scavenging Activity of Lupeol Isolated from the Methanol Leaf Extract of *Crateva adansonii* Oliv. (Capparidaceae), Int. J. Pharmacog. Phytochem. Res. 8, 3, (2016) 419-426.

46. L.K. MacDonald-Wicks, L.G. Wood, M.L. Garg, Methodology for the determination of biological antioxidant capacity in vitro: a review. J. Sci. Food Agric. 86 (2006) 2046–2056.

47. W.K. Wang, H.S. Park, I. Ham, M. Oh, H. Namkoong, H.K. Kim, D.W Hwang, et al., Natural compounds, fraxin and chemically structurally related to fraxin protect cells from galo-oxidative stress. Exp. Mol. Med. 37 (2005) 436–446.

48. S.R. Grobler et al., Cytotoxic Effect of Chitosan-H, Resveratrol, β-Carotene and Propolisand their Chitosan Hydrogels On Balb/C Mouse 3T3 Fibroblast Cells. Int. J. Dentistr.y Oral. Sci. 1(2) (2014) 10-14.

ACCEPTED MANUSCRIPT

49. E. Szymanska, K. Sosnowska, W. Miltyk, M. Rusak, A. Basa, K. Winnicka, The Effect of Glycerophosphate Crosslinking on Chitosan Cytotoxicity and Properties of Hydrogels for Vaginal Application. Polymer. 7 (2015) 2223–2244; doi:10.3390/polym7111510.

Figure caption

Fig. 1. Possible reaction in crosslinking/Polymerization; [A] Schiff base formation b/w CH & GLUT; [B] Acetal formation b/w CH & GLUT; [C] Schiffbase formation b/w GEL & GLUT; [D] Whole crosslinking reaction. CH: Chitosan; GLUT: Glutaraldehyde; GEL: Gelatin

Fig. 2. SEM images of a) chitosan/gelatin film, b) lupeol loaded chitosan/gelatin film

Fig. 3. FTIR Spectrum of (A) Chitosan, (B) Gelatin and (C) Chitosan/Gelatin Hydrogel

Fig 4: DSC thermograms of (a) Lupeol, (b) Chitosan, (c) Gelatin, and (d) hydrogel

Fig.5. (a). Cell viability of different dilution of CGH films. DMEM media 100% used as control. **(b).** Box & Whisker plot of the % cell viability of NIH/3T3 fibroblast cells.

Table caption

Table 1: Different hydrogel film formulations and their characterization parameters

Table 2: Absorbance and % antioxidant activity of different reaction mixture in DPPH assay & NBT assay

Table 3: Determination of zone of inhibition of *P. aeruginosa* (MTCC 424)

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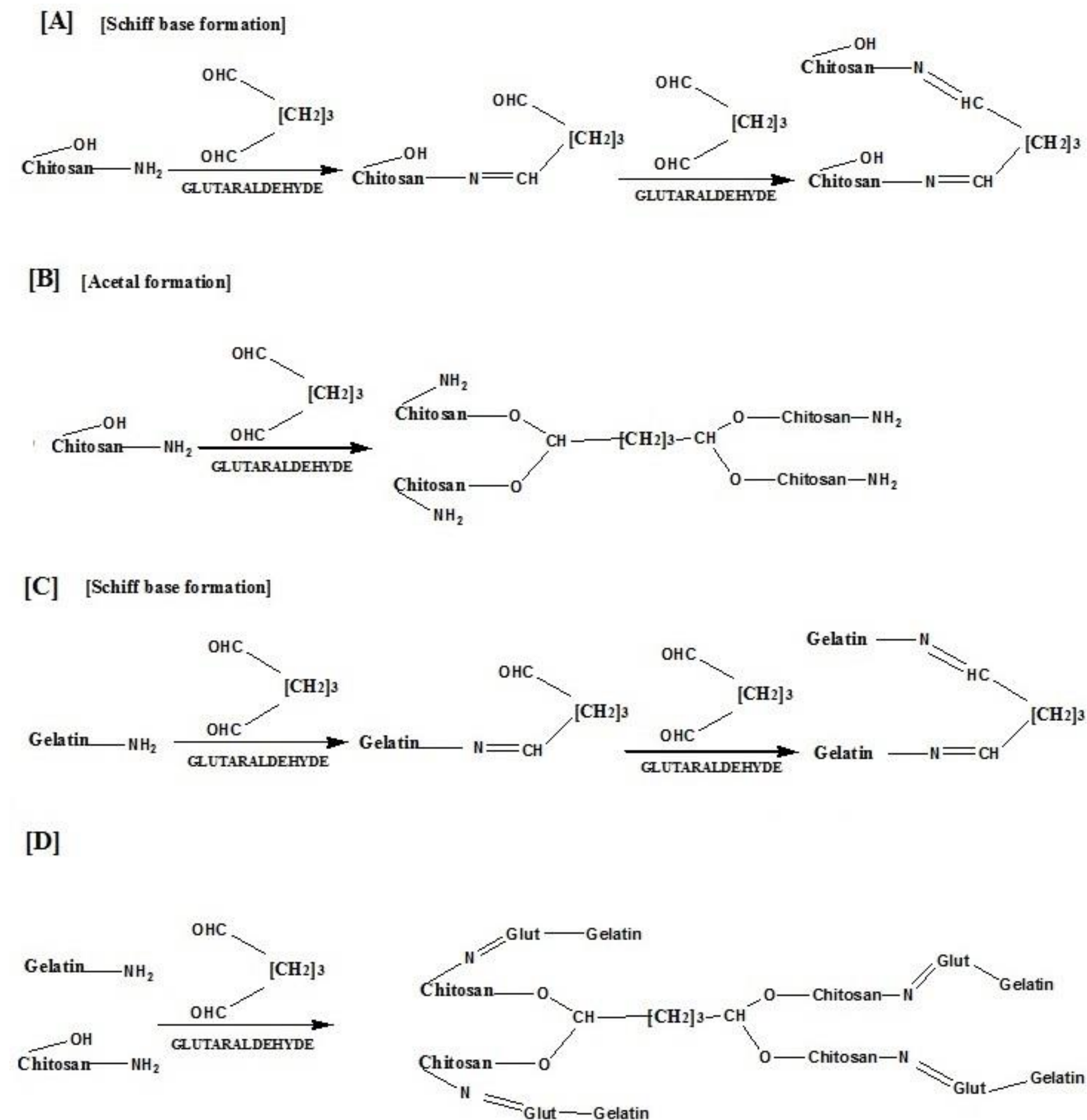
Figr-1



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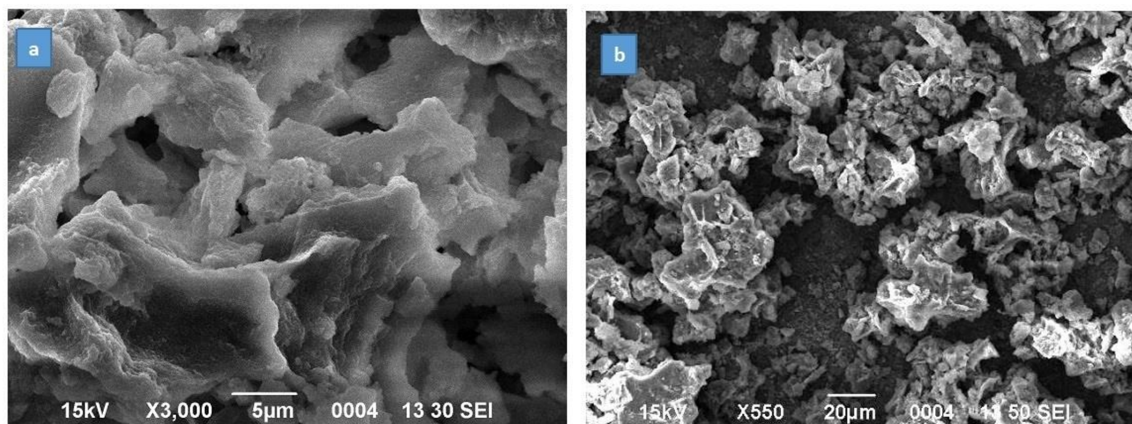
Figr-2



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Figr-3

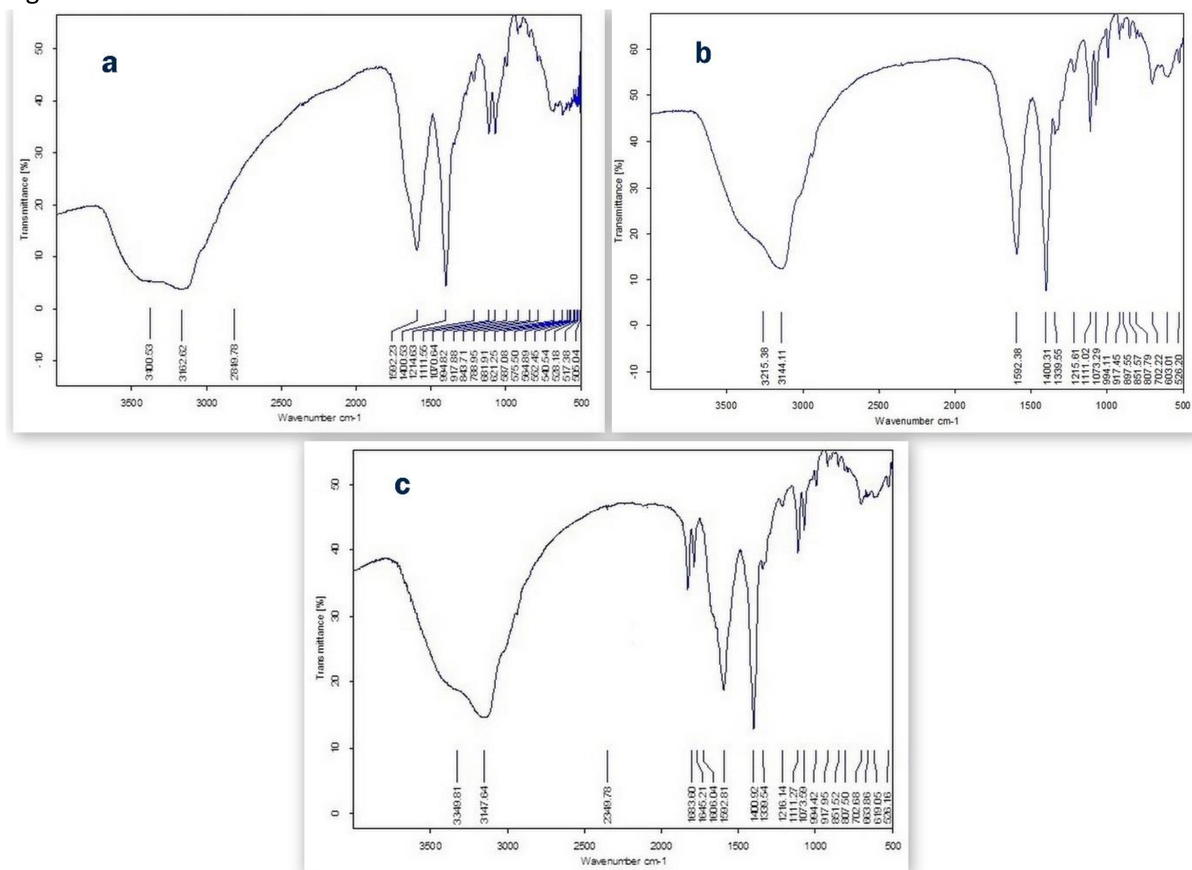

Fig. 3 FTIR Spectrum of (A) Chitosan, (B) Gelatin and (C) Chitosan/Gelatin Hydrogel

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Figr-4

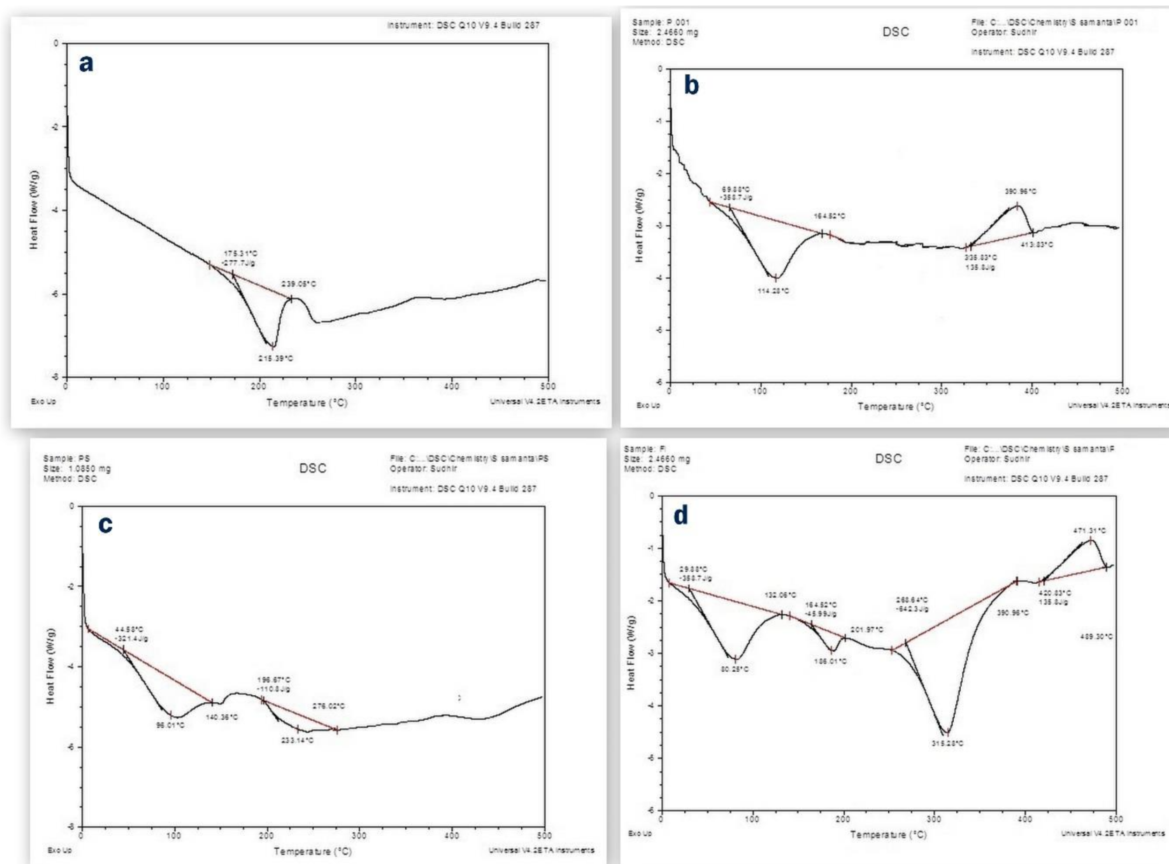

Fig.4: DSC thermograms of (a) Lupeol, (b) Chitosan, (c) Gelatin, and (d) hydrogel

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Figr-5

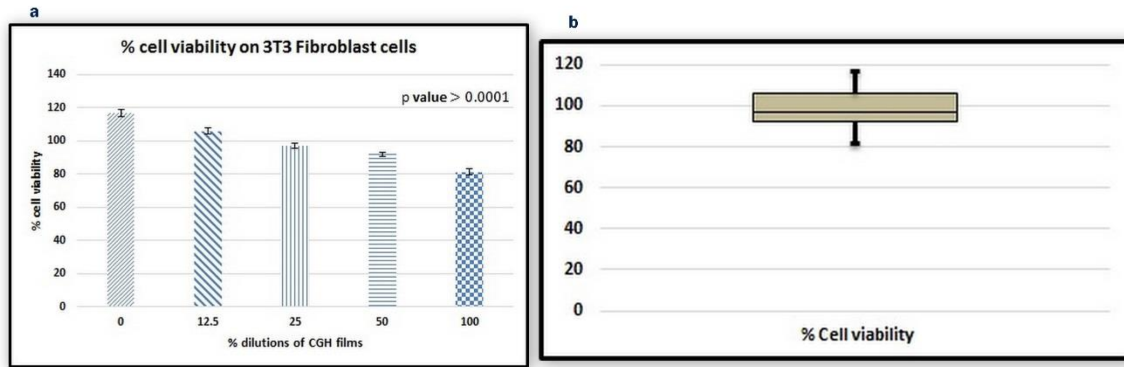

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Table 1: Different hydrogel film formulations and their characterization parameters:

Film Ingredients		R*	Hydrogel Film	Characterization Parameters**			
Chitosan Vc (ml)	Gelatin Vg (ml)			Thickness (mm)	Weight (gm)	WVTR (g/m2/day)	%EWC
10	90	0.1	**F-1**	0.719±0.004	0.38±0.015	1975± 33.75	77.5
30	70	0.3	**F-2**	0.623±0.001	0.36±0.013	1545 ± 42.6	77.71
50	50	0.5	**F-3**	0.452±0.001	0.26±0.013	2228 ± 31.8	85.40

*The mixture ratio was calculated as $R = \frac{Vc}{Vc + Vg}$. ** Values are (mean ± SD) (n = 3)

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Table 2: Absorbance and % antioxidant activity of different reaction mixture in DPPH assay & NBT assay

Lupeol (Pure)	**Concentration (µg/mL)**					
	500 µg/mL	**50 µg/mL**	**25 µg/mL**	**5 µg/mL**		
Absorbance	0.019	0.032	0.045	0.064		
% antioxidant activity	94.41	90.58	86.76	81.17		
Hydrogel film	**Time (Hours) DPPH Assay**					
	1h	**2 h**	**3 h**	**4 h**	**5 h**	**6 h**
Absorbance	0.069	0.064	0.062	0.044	0.040	0.032
% antioxidant activity	79.82	81.28	81.81	87.05	88.20	90.61
Hydrogel film	**Time (Hours) NBT Assay**					
	1h	**2 h**	**3 h**	**4 h**	**5 h**	**6 h**
Absorbance	0.098	0.087	0.064	0.049	0.038	0.029
% antioxidant activity	66.20	70	77.90	83.10	86.89	90.00

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Table 3: Determination of zone of inhibition of *P. aeruginosa* (MTCC 424)

S. No.	Pure Lupeol concentration	Zone of inhibition (mm)	Release from Film Samples	Zone of inhibition (mm)
1.	W-1 (0µg/ml)	0±0.0	W-7 (1 hr)	16.5±0.0
2.	W-2 (5µg/ml)	4±0.6	W-8 (6hr)	17.2 ±0.5
3.	W-3 (10µg/ml)	6±0.5	W-9 (8 hr)	21.0±2.0
4.	W-4 (15µg/ml)	9±1.0	W-10 (10 hr)	22.5±1.0
5.	W-4 (20µg/ml)	11±1.2	W-11 (12 hr)	23±0.5
6.	W-6 (25µg/ml)	12±1.5		