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Evaluation of Diabetic Wound Healing Activity by Topical Application of Gallic Acid Loaded Chitosan Composite Scaffold on Streptozotocin-Induced Diabetic Rats

Darshan K [1], **Bhargav Bhongiri** [1], ***Vadivelan Ramachandran** [1], **Tharani Mohanasundaram** [1]

[1]Department of Pharmacology, JSS College of Pharmacy, JSS Academy of Higher Education & Research, Ooty, Nilgiris, Tamil Nadu, India

Abstract

Diabetic foot ulcers are complicated sores that interfere with the skin's functional activity and, more critically, the essential role of skin cells. Gallic acid is a plant-derived polyphenol-rich substance that has been linked to health benefits such as antioxidant and anti-inflammatory action. The research will focus on generating and improving gallic acid-loaded chitosan-gelatin composite scaffolds for diabetic wound healing. The scaffolds are then evaluated for their physicochemical characteristics, cell viability is determined using the in-vitro cytotoxicity technique, and putative wound healing activity in full-thickness excision wounds in an in-vivo model of streptozotocin-induced diabetic rats is investigated. The freeze-drying process was utilized to prepare the formulation (Scaffolds). Porosity, water absorption, and matrix degradation Preformulation experiments were carried out (69.8%, 32.07%). The in-vitro drug release research indicated that 52.55% of the medication is released after 48 hours, while the in-vitro cytotoxicity study found that the IC50 was 32.1g/mL. in-vivo studies have been conducted for Dermal irritation. By injecting STZ 60mg/dL in a group of rats and establishing diabetic wounds, researchers were able to examine wound contraction, hexosamine content, and hydroxyproline content. The studies were completed and reported on.

Keywords: Diabetic foot ulcers, Gallic acid, Chitosan-gelatin scaffolds, Streptozotocin, Freeze-drying method

1. Introduction

Diabetes foot ulcers (DFU) are among the most common complications in diabetic patients, leading to amputation and psychological distress. This mini-review covered the general physiology of ulcer healing as well as the pathophysiology of DFU and therapies. Only a few treatments have been sanctioned and numerous compounds are now being tested in various stages for the prevention and treatment of DFUs (Okonkwo and Dipietro 2017). In the year 2017, the International Diabetes Federation (IDF) predicts that 425 million individuals suffer from this chronic metabolic disorder throughout the world, in which at least one limb is removed every 30 seconds due to chronic ulcers. For the application of wound healing, scaffolds such as 3d-porous matrix, Hydrogels, Microspheres and Nanofibers have been created. The capacity of the designed tissue scaffolds for the absorption of a large volume of the fluid while keeping the wound bed wet is critical for

*Corresponding author

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skin regeneration and wound healing (Zhang et al., 2017). Standard cotton pads fluid retention capacity is typically restricted to 67% of the dry weight. A good absorbent scaffold is required to limit replacement of the pad during the surgical intervention as well as for the chronic wounds that will produce large volumes of the exudates. To develop and optimize the gallic acid loaded chitosan-gelatin composite scaffolds for diabetic wound healing.

2. Materials

Gallic acid, MES buffer (2-(N-morpholino) ethane sulfonic acid hemi sodium salt, EDC (N-(3-Dimethylamino propyl-N-ethyl carbodiimide and NHS (N-hydroxy succinimide was obtained from Sigma labs, Mumbai, India. Potassium dihydrogen ortho phosphate, Sodium hydroxide pellets were procured from SD fine chemicals, Mumbai, and other chemicals reagents used were of analytical grade.

3. Methodology

3.1 Preformulation Study (Bhongiri et al., 2022)

Preformulation may be described as a phase of the research and development process where the formulation scientist characterizes the physical, chemical and mechanical properties of a new drug substance, in order to develop stable, safe and effective dosage forms (Kesharwani et al., 2017). Ideally, the preformulation phase begins early in the discovery process such that appropriate physical, chemical data is available to aid in the selection of new chemical entities that enter the development process. During this evaluation possible interaction with various inert ingredients intended for use in final dosage form are also considered (Patel, 2020).

3.2 Development of Calibration Curve

For the preparation of the stock solution 10mg of Gallic acid was dissolved in 15ml of ethanol and made up to 100mL with distilled water. Serial dilutions were prepared from the stock solution i.e., 1mL (10mcg), 2mL (20mcg), 3mL (30mcg), 4mL (40mcg), 5mL (50mcg) by using distilled water. The λ max of the drug was obtained by scanning one the dilutions under UV- Spectrophotometer. The λ max is used for the determination of absorbance for all the solutions against the blank solution. Standard curve was plotted by taking concentration on the x-axis and the intercept on y-axis and the value of the intercept and slope are obtained.

3.3 Solid Dispersion Technique

Drug and mixed carrier dispensed in a beaker and placed in a water bath held at 60 c. Drug and mixed carrier dissolved in defined amount of absolute ethyl alcohol. The solution is then transferred to rotary evaporator and solvent evaporated under vacuum. The preparation is then cooled immediately at 20℃ and solidified for 12hrs. Solidified mass is stored in thermostatic Air- blower- driven closet and then pulverized by a pestle. The powder is passed through 0.154mm sieve and collected (Shuai et al., 2016).

3.4 Preparation of Composite Scaffold

Chitosan solution (2%) was prepared by dissolving chitosan (2g) in 1% acetic acid solution (100ml) at the room temperature. The composite scaffolds were prepared by dissolving 5mg and 10mg Gallic acid drug in chitosan solution. These solutions kept under magnetic stirrer until opaque aqueous solutions were obtained. The entrapped bubbles in the solutions were removed by keeping them in sonicate for 5 minutes, then these solutions were poured in the petri-dishes and froze in the deep freeze at -60℃ for 24 hours followed by lyophilization for 72 hours to obtain the Gallic acid composite-scaffolds.

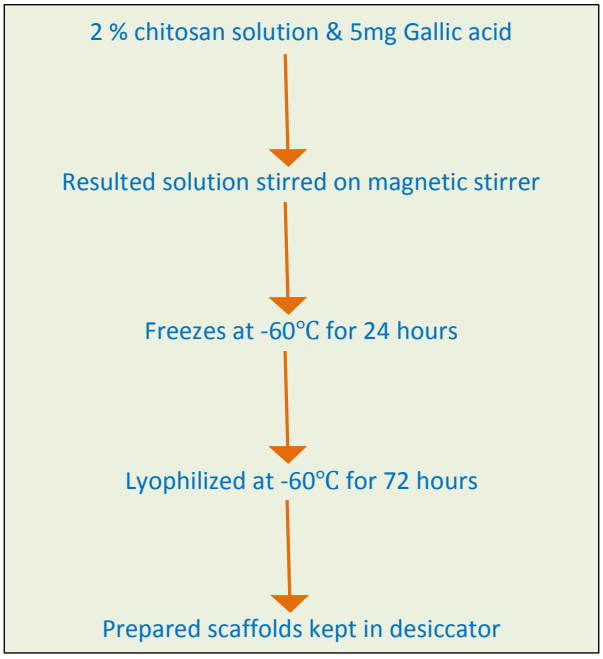


Fig. 1 Formulation of scaffolds

3.5 Cross-linking

To enhance mechanical properties and prevent cell-mediated contraction, a variety of physical and chemical cross-linking techniques have been applied to the scaffolds. Weighed 0.488 gm of MES (2-(N-morpholino) ethane sulfonic acid) and dissolved in 50 ml of distilled water. The pH of the buffer solution was measured using pH meter. Weigh accurately 50mg of the drug loaded scaffold. Take 20ml of the MES buffer in a beaker and immerged the scaffold in it for 30 min. Take 19.5 ml of the MES buffer in another beaker add 0.1264 gm of EDC (1-ethyl dimethyl aminopropyl carbodiimide) and 0.014 gm of NHS (N-hydroxysulfosuccinimide) and mixed well. Dip the soaked scaffold in the buffer mixture for 4 hrs for cross linking. Filter out the cross-linked Scaffold and wash twice with 0.1M NaH_2PO_4 for 1hour. Wash it again with 1 M NaCl and 2M NaCl for 2 hrs and 1 day respectively (Kaparekar et al., 2020).

3.6 Characterization of the Composite Scaffold

3.6.1 Porosity

The porosity of the scaffolds can be determined by liquid displacement method. In this method ethanol was used as displacement liquid as it can effortlessly penetrate into the pores of scaffolds, did not stimulate contraction and swelling of scaffolds. Gallic acid containing scaffolds with characteristic collagen is subjected for determination of porosity. Ethanol displacement method is used to determine the pore volume (Vp) and geometric volume (SV) of the scaffolds disc are measured by determining the diameter and heights. Air bubbles are removed by immersing the weighed scaffolds (Ww) in absolute ethanol at room temperature and then kept in desiccators under reduced pressure for five minutes.(Balducci, Stefano, Sacchetti, Massimo, Haxhi, Jonida, Orlando, Giorgio, D'Errico, Valeria, Fallucca, Sara, Menini, Stefano, Pugliese 2014)

Samples were taken out in subsequent manner, then wiped with filter paper to remove excess of ethanol and weighed instantly (Wd). Porosity of scaffolds is calculated according to the following equation.

$$\boldsymbol{Porosity}(\%) = [\boldsymbol{Ww} - \boldsymbol{Wd}]/\boldsymbol{SV} * \mathbf{100}$$

Where SV is the scaffold volume; Ww and Wd are the wet and initial dry weights of the scaffolds, respectively.

3.6.2 Matrix Degradation

To measure the biodegradability, the initial weight of scaffold was measured as (Wo) and was immersed in phosphate buffer solution (pH 7.4). Then the scaffold was kept at 37°C in incubator and the measurements were taken up to seven days by determining the final weight (wt). Finally, the percentage loss was determined (Ni et al., 2006).

$$\boldsymbol{Degradation}\ \% = \frac{[\boldsymbol{W0} - \boldsymbol{Wt}] \times \mathbf{100}}{\boldsymbol{Wo}}$$

3.6.3 Water Absorption Test

The scaffolds were weighed as dry weight (Wo). The scaffold sample then incubated in phosphate-buffered saline (PBS) at pH 7.4 at 37°C for 2 hours. The swollen scaffold then weighed after removing the excess water by blotting with filter paper. Then proceed for lyophilization and reweighing of scaffold sample. The water content of scaffold sample calculated using the following formula.

$$\boldsymbol{Ead = [We - Wo]/Wo * 100}$$

Where *ad* is the percentage water adsorption of CS scaffold at equilibrium. and o represent the weight of the CS scaffold at equilibrium and initial weight of the CS scaffold, respectively.

3.7 In Vitro Drug Release Study

The composite scaffolds (3 × 3cm) were immersed in 20 mL phosphate buffer solution having pH 7.4 at 37∘C. The supernatant was taken out periodically and replaced by equivalent volume of BPS. The drug release is determined by UV-visible spectrometer at 250 nm by using Gallic acid standard curve in ethanol. The percentage of Gallic acid released was determined.(Weng, Tong, and Chow 2020)

3.8 In Vitro Cytotoxicity Study

MTT [(3-(4,5 dimethylthiazol-2yl) -2,5-diphenyltetrazolium bromide)] assay was used to assess the biocompatibility of the prepared scaffolds. Scaffolds of standard dimensions (15 mm diameter) were sterilized for 30 min in 75% ethanol, and then washed with sterile water for 5 min. One scaffold was then placed in each well of the 24 well plates with 2 mL of Dulbecco's modified Eagle's medium. To this 24 well plate, 3T3-L1 cells were added. Test was performed for 72 h, wherein both control and sample media were maintained for proper comparison (Abd El-Aziz et al., 2021).

3.9 In-vivo Wound Healing Study

3.9.1 Acute Dermal Irritation Test

Acute dermal irritation test will be performed in 20 weeks old albino rabbit weighing 4- 5Kg. 24 h prior the test fur will be removed on the back of the rabbit carefully to avoid abrading the skin. Scaffold prepared will be applied to a small area of skin (5 cm x 5 cm) and untreated skin area of the test animal will be used as a control. At the end of the exposure period (72 h), the residual formulation will be removed using gauze and water without altering the existing response or integrity of the epidermis. Scoring for erythema and oedema will be given according to OECD 404 from 0 to 4. 0 scores for erythema or oedema, 1 score for very slight erythema or oedema, 2 scores for well-defined erythema or oedema, 3 scores for moderate and 4 scores for severe will be given.

Table 1 Grading of skin reactions (OECD 404)

Erythema and Eschar Formation

No erythema	0
Very slight erythema (barely perceptible)	1
Well defined erythema	2
Moderate to severe erythema	3
Severe erythema (beef redness) to eschar formation preventing grading of erythema	4

Maximum possible: 4

Oedema Formation

No oedema	0
Very slight oedema (barely perceptible)	1
Slight oedema (edges of area well defined by definite raising)	2
Moderate oedema (raised approximately 1 mm)	3
Severe oedema (raised more than 1 mm and extending beyond area of exposure)	4

Maximum possible: 4

3.9.2 Diabetes Induction and Wound Creation

Healthy, adult male Wistar rats (200–240 g) were utilized for the in vivo wound studies. They were housed in standard polycarbonate cages with ad lib access to standard chow, water and maintained on a 12:12-h light, dark cycle in a climate-controlled room. Following 10

days of acclimatization, a single injection of STZ (60 mg kg−1) in citrate buffer solution (0.1 M, pH 4.5) was intraperitoneally administered to initiate the induction of diabetes. After two days of STZ injection, animals with blood glucose levels ≥300 mg/dL (Glucometer; AccuSure, Taiwan) were kept under observation for 7 more days and only those that consistently demonstrated elevated blood glucose levels were selected for the subsequent procedures. A wound measuring 2 × 2 cm2 (≈400 mm2) was created on the dorsal thoraco-lumber region of the diabetic rats under ketamine and xylazine anaesthesia (100 mg/kg and 10 mg/kg, respectively). The wounds were neither dressed nor covered. After anaesthesia recovery, the animals were housed individually and closely monitored (61, 62).

The animals were grouped into three groups. Group 1 is treated with sterile gauze (control), group 2 is treated with 2% Chitosan scaffold (Cross-linked scaffold without Gallic Acid) (reference group) and group 3 is treated with 5mg Gallic Acid-Chitosan Cross-linked scaffold (Test group). All animal experiments were approved by Institutional Animal Ethical Committee, J.S.S. College of Pharmacy, Ooty (Proposal no. JSSCP/OT/IAEC/13/2021-22). Percentage wound contraction was measured using the formula.

$$\%\,\boldsymbol{Wound\ contraction} = \frac{wound\ area\ on\ day\ 0 - wound\ area\ on\ particular\ day}{wound\ area\ on\ day\ 0} \times 100$$

3.10 Biochemical Analysis

Granulation tissues collected from all the experimental animals on 15th day were used for the estimation of Hydroxyl proline and Hexosamine estimation.

3.10.1 Hydroxyproline Estimation

The presence of collagen in the granulation tissues was estimated using the amount of hydroxyproline (an imino acid) released from the samples, a key indicator of collagen.(Morey et al. 2019) The tissue samples were washed in physiological saline and defatted using a chloroform- methanol mixture in the ratio 2:1 and frozen in acetone. The frozen tissues were hydrolysed in 3 ml of 6 N HCl at 110 °C for 20 h. The samples were allowed to dry in a hot air oven and the residue obtained was dissolved in distilled water. Hydroxyproline is oxidized by chloramine T (Sodium p-toluene sulfonchloromide) to give pyrrole carboxylic acid, which is further oxidized by PDAB (p-dimethyl amino benzaldehyde) to give cherry red colour which was measured at 557 nm.(Rohani and Parks 2015).

3.10.2 Estimation of Hexosamine

The amount of hexosamine present in the granulation tissues was estimated with a procedure of sample preparation similar to that of hydroxyproline estimation. Hexosamine reacts with acetyl acetone (2,4- pentane diox) in the presence of an alkali to form cyclic oxazole or pyrrole that is coupled with Ehrlich's reagent to form stable chromophore. The absorbance was measured at 530 nm. The content of hexosamine was estimated in comparison with a standard curve and content is expressed as mg/g dry tissue weight.(Martins, Caley, and O'Toole 2013)

3.11 Histopathological Studies

At the end of the study, animals from each group were sacrificed with anaesthesia overdose to collect the granulation/healing tissue. It was preserved in 10% formalin for histopathological evaluation. Haematoxylin and eosin (H&E) staining was used to determine the gross morphological changes at the wound site. The granulation/healing tissues fixed in 10% formalin were embedded in paraffin. Rotary microtome (Leica Biosystems, India) was used to obtain 5µm thick tissue sections and stained with H&E, as per the standard method and visualized under a light microscope (Olympus CX31, Japan) at 40X magnification.

3.12 Statistical Analysis

The results were statistically analysed by GraphPad prism version 8.01 using a one-way ANOVA followed by Tukeys multiple comparison test. The data were expressed as a mean ± standard deviation. In all of the evaluations, $P < 0.05$ was considered statistically significant.

4. Results and Discussion

4.1 Development of Calibration Curve

Calibration curve of the drug was developed in distilled water and it was found out that the perfect correlation was observed. From the regression value (R2 = 0.9717) it was concluded that the perfect linearity between the concentration and absorbance was observed, when the concentration range was from 10µg/ml to 50µg/ml.

Table 2 Calibration curve of Gallic acid in distilled water at λ max= 250 nm

S. No	Concentration	Absorbance
1	10	0.002
2	20	0.118
3	30	0.326
4	40	0.354
5	50	0.380

Table 3 Percentage degradation data of Gallic acid loaded chitosan scaffolds

Degradation Time (Days)	% Degradation
1	6.1
2	11.3
3	16.7
4	21.9
5	26.9
6	32.7
7	39.5

4.2 In Vitro Drug Release Studies

Drug release profiles of the Gallic acid loaded chitosan scaffold were studied over a period of 48h with aliquots taken from the tubes at 60 minutes intervals initially for the first 3 hours, followed by every 3 hours, 6 hours and then 12 hours interval thereafter. There was a slower release in all formulation followed by a sustained release and then a plateau. The drug release rate is more as it has more drug entrapped in the scaffold.

Table 4 In vitro % drug release study of Gallic acid loaded chitosan scaffolds

Time	% Drug Release
0	0
1	7.38
3	17.12
6	26.88
12	33.47
24	40.0
48	52.55

4.3 In Vitro Cytotoxicity Assay

The percentage growth inhibition was calculate using the following formula and concentration of drug or test samples needed to inhibit cell growth by 50% values were generated from the dose response curve for each cell line. The IC50 of Gallic acid was found to be 32.1 µg/mL (Fig. 1, 2, 11).

$$\%\ Cell\ Viability = \frac{Mean\ OD\ of\ individual\ test\ group}{Mean\ OD\ of\ Control\ Group} * 100$$

4.4 Acute Dermal Irritation Test

Rabbit did not show any clinical signs any overt signs of toxicity from the first day until the end of the experiment. The skin of the animals appeared normal, and no erythema or edema was noted. The locomotor behaviour was also normal, and there were no signs of toxicity detected in the treated rabbit.

Table 5 Acute Dermal Irritation Study of Scaffold in Rabbit

Skin reaction	Erythema			Edema		
Observation time (Hours)	24	48	72	24	48	72
Total score	0	0	0	0	0	0
Mean score [a]	0	0	0	0	0	0
Total of mean score	0					
PII [b]	0					
Remarks	Non-irritating					

PII, primary irritation index

1. Dermal responses were scored according to OECD guidelines 404.
Mean score of dermal responses = (total score of erythema formation + total score of edema formation)/3.
2. PII = (mean score at 24 hours + mean score at 48 hours + mean score at 72 hours)/3

4.5 Wound Contraction Study

Wound contraction was analyzed in each group as a percentage of the reduction in wounded area at day 3, 7, 11 and 15 (Fig. 3, 4, 10). In the present study, GA-CS scaffold treated wounds contracted significantly ($p<0.001$) faster than the wounds from the control and CS scaffold treated groups. The mean percentage of wound contraction of the GA-CS scaffold treated group was significantly (84.92±0.60 % at day 15, $p<0.001$) higher compared with those of the control (41.26±0.51% at day 15, $p<0.01$) and scaffold-treated (53.21±0.42% at day 15, $p<0.01$) groups after wound creation. The effect of scaffold on wound contraction is shown in the (Fig. 5, 6, 9).

Table 6 Effect of scaffold on wound contraction

Groups	Wound contraction (%)			
	Day 3	Day 7	Day 11	Day 15
Control	12.77±0.55	16.13±0.27	23.05±0.28	41.26±0.51
CS Scaffold	16.52±0.55*	21.68±0.30*	39.53±0.43*	53.21±0.42**
GA-CS Scaffold	24.02±0.49$^{*\#}$	28.25±0.38$^{*\#}$	64.92±0.46$^{*\#}$	83.82±0.61$^{*\#}$

4.6 Effect of Scaffold on Hydroxyproline Content

Hydroxyproline content was found to be significantly higher in the CS scaffold treated group compared with the control group ($p<0.05$). However, the hydroxyproline content of GA-CS scaffold treated group was found to be higher than in CS-scaffold treated group ($p<0.05$)

4.7 Effect of Scaffold on Hexosamine

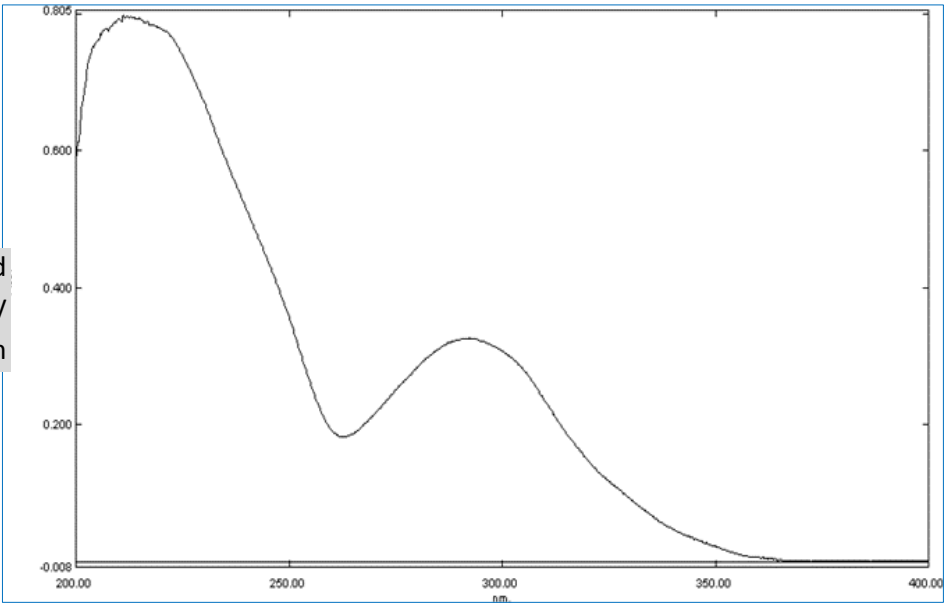
Hexosamine content was found to be significantly higher in the CS scaffold treated group compared with that in the control group ($p<0.05$). However, the hexosamine content of GA-CS scaffold treated group was found to be higher than in CS-scaffold treated group ($p<0.05$) (Fig. 7, 8).

Table 7 Effect of scaffold on Hydroxyproline and Hexosamine content

Groups	Hydroxyproline content (µg /mg dry tissue)	Hexosamine content (mg/100 mg dry weight)
Control	7.27±0.78	0.24±0.05
CS Scaffold	10.94±0.48*	0.39±0.03*
GA-CS Scaffold	17.09±0.55 *$^{\#}$	0.53±0.07 *$^{\#}$

Data are expressed as means ± SEM (n = 6).
*p < 0.05 vs control and #p < 0.05 vs. CS Scaffold

Fig. 2 Maximum wavelength of Gallic acid in distilled water was measured from UV 400-200nm



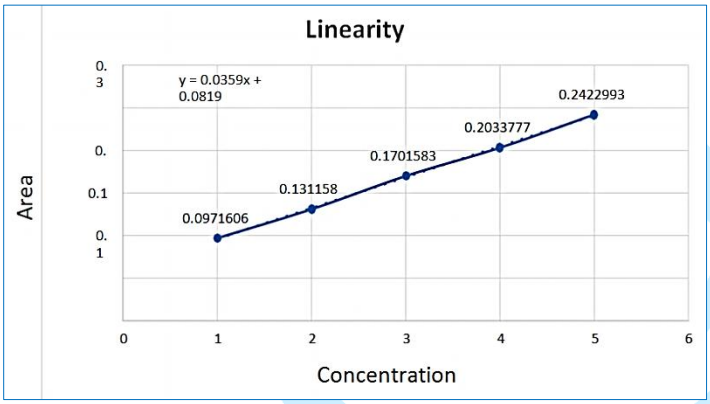


Fig. 3 Calibration curve of Gallic acid

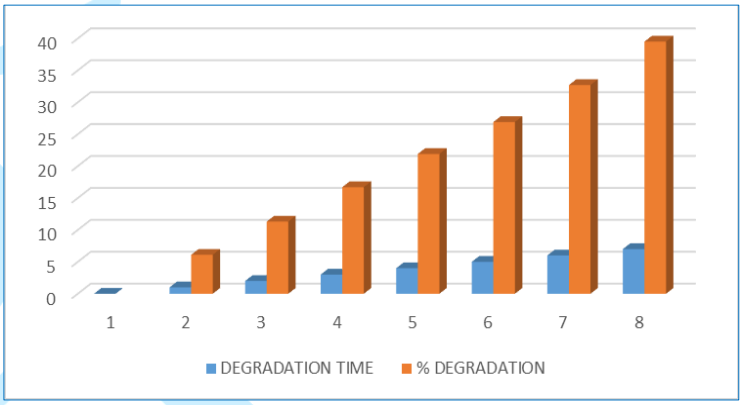


Fig. 4 Matrix Degradation Study of Gallic acid loaded chitosan scaffolds

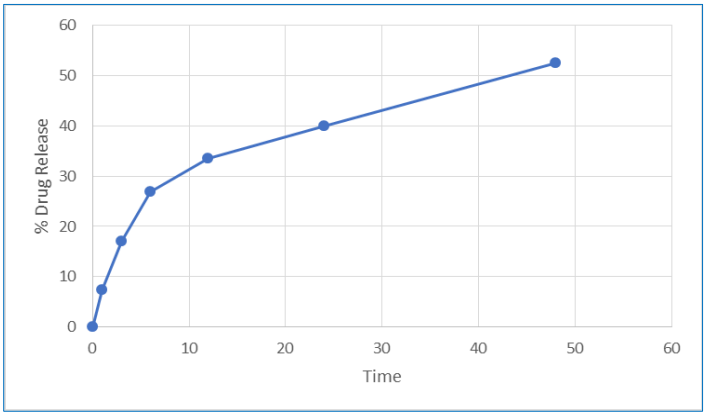


Fig. 5 In vitro % drug release study of gallic acid loaded chitosan scaffolds

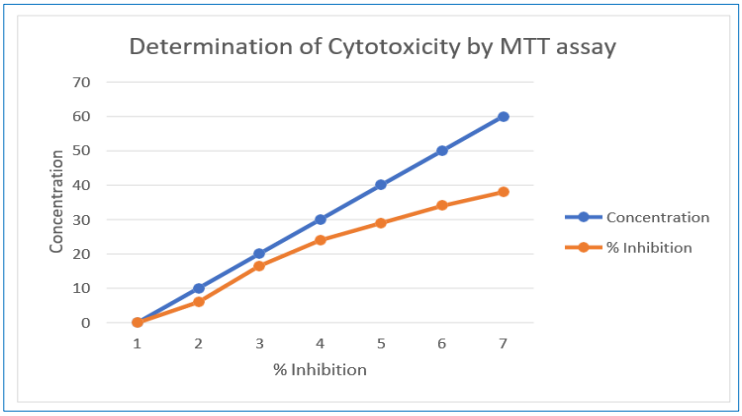


Fig. 6 Percentage inhibition of Gallic acid loaded Scaffold

Fig. 7 Dermal irritation study of scaffold in Rabbit

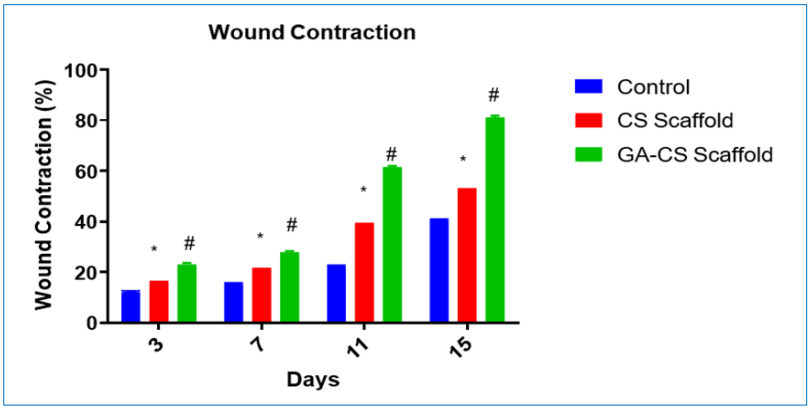


Fig. 8 Effect of scaffold on wound contraction

7, 11 and 15 post-wounding. Data are expressed as means ± SEM (n = 6)

*p < 0.05 vs control and #p < 0.05 vs. CS Scaffold

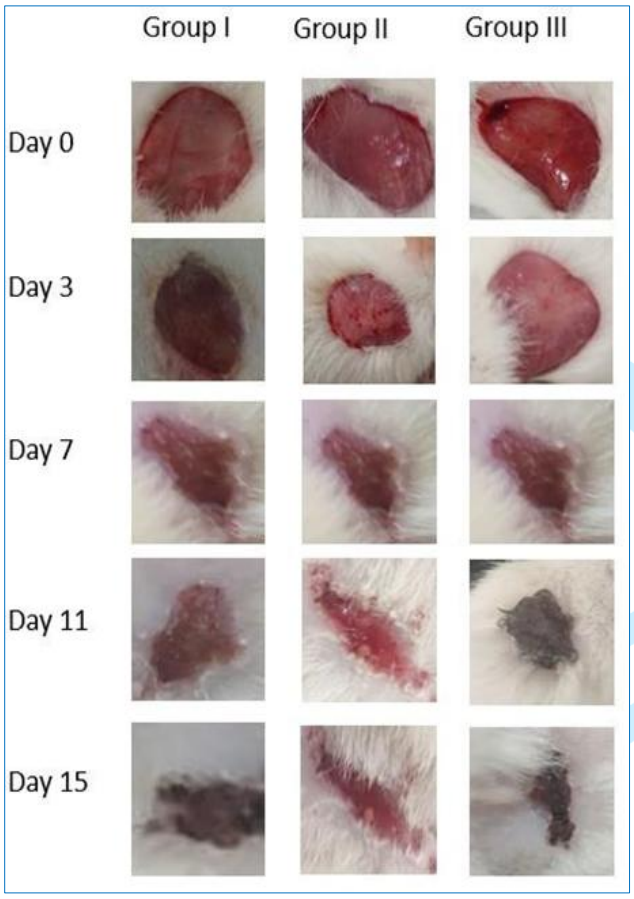


Fig. 9 Digital photographed images of wound contraction

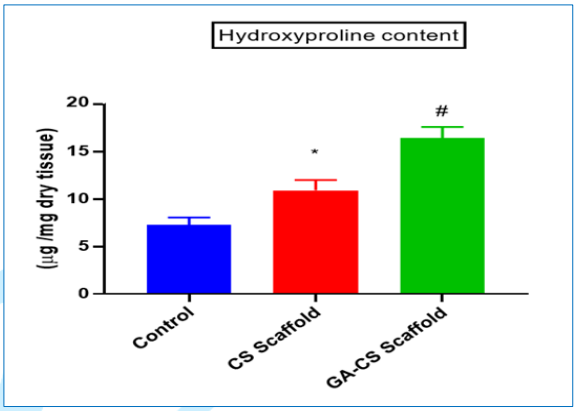


Fig. 10 Effect of scaffold on Hydroxyproline content

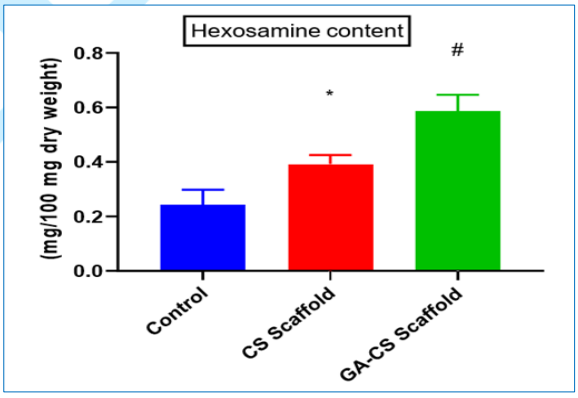


Fig. 11 Effect of scaffold on Hexosamine content

5. Conclusion

In exhibiting anti-inflammatory, anti-oxidant, anti-diabetic activities, Gallic acid was found to show quicker wound healing activity since Chitosan polymer is responsible for processing of the wound healing through a series of inter-reliant and corresponding stages. Due to this, there occurs a variety of cellular and matrix components that act together to reintegrate the damaged tissue and in replacing lost tissue. The solid dispersion technique was the first improved method to validate the solubility of the drug. The following tests have been performed which are mentioned below:

- Using UV-Vis Spectrophotometer, the calibration curve was obtained from which the r^2 was found to be 0. 9994. Preformulation studies of porosity, water absorption, matrix degradation was performed (69.8 %, 32.07%). From the in-vitro drug release study, 52.55% of drug is release at 48 hours and the IC50 for the in-vitro cytotoxicity study was found to be 32.1µg/mL. In-vivo studies of dermal irritation test have been performed. Examination on wound contraction, hexosamine content and

hydroxyproline content have been performed by inducing STZ 60mg/dL in a group of animals and creating diabetic wounds.

- The study was aimed at promoting wound healing in diabetic rats by fabricating Gallic acid loaded Chitosan scaffold. From the study, the drug solubility can be increased by using the solid dispersion technique. So, preparation of Chitosan scaffolds (CS) and Gallic acid chitosan scaffolds (GA-CS) were initiated since scaffolds have a good porous nature, water absorption and matrix degradation whereas Gallic acid loaded scaffold exhibited enhanced viability of the cell. Upon subjecting the rabbit to irritation test, it was observed that there was no formation of erythema or oedema. From the preliminary studies, it was observed that Gallic acid loaded chitosan scaffolds can be preferred as a potential target in the treatment of diabetic wounds since Gallic acid showed a higher percentage of wound closure, collagen content and hexosamine content than compared to the blank scaffold and control groups.

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Declaration of Conflict

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper

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