

Formulation optimization and wound healing activity of *vitex trifolia L* leaf extract loaded chitosan hydrogel film on hyperglycemic rats

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ABSTRACT

Decreased blood supply, high blood sugar level, and a possibility of bacterial infection in diabetic wounds pose risk for limb loss and mortality. Therefore, proper wound care is needed to improve the quality of life of diabetic patients. *Vitex trifolia L* (Legundi) extract is reported to have antibacterial and antioxidant activity that enhances cell proliferation and migration. The antimicrobial and hemostatic properties of chitosan film are viewed as an ideal material for enhancing wound healing. The film should retain its integrity and flexibility while used on the skin, therefore chitosan was combined with PVA and PVP K30 to improve its quality. The objectives of this study were to optimize the concentration of chitosan, PVP K30, and PVA towards the Legundi extract film properties, and to evaluate its wound healing activity on hyperglycemic mice using an incision wound model. In this study, the film's compositions were optimized using a simplex lattice design, and the effects of its components on their characteristics, such as thickness, weight, folding endurance, swelling rate, and swelling index, were evaluated. BALB/c Mice were divided into three groups (Group I, Group II, Group III) which were treated with normal saline, placebo film, and Legundi film respectively once daily for 8 days. The result suggested that chitosan and PVA were responsible for affecting the film's thickness, weight, and folding endurance, whilst PVP K30 was the dominant factor in increasing the swelling index and rate of the film. The optimum formula of Legundi extract films consists of 1.15 % (b/v) chitosan, 1.25 % (b/v) PVP K30, and 1.6 % (b/v) PVA. The animal treated with Legundi extract film have higher wound closure compared to the control and placebo group four days after wound incision ($p < 0.05$). Thus, Legundi extract film was a potential dressing to treat a diabetic wound.

Keywords: *Vitex trifolia L.*, hydrogel film, chitosan, hyperglycemic, wound healing

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INTRODUCTION

Foot ulcers are the biggest contributor to medical costs for people with diabetes mellitus. In contrast to normal wounds, the high blood sugar level in diabetic wounds makes them suitable for bacterial growth, and therefore patients most likely will have a bacterial infection specifically from *Staphylococcus aureus* and *Pseudomonas aeruginosa* (Kartini et al., 2018; Kavitha, 2014). This condition prolonged the wound-healing process and increase the significant risk of morbidity, amputation, and mortality Kavitha, 2014. In 2013 around 6.9% of Indonesian people (age > 15 years) suffer from diabetes mellitus, 15% of them undergo diabetic foot ulcer, and 15-27% of patients with diabetes require lower limb amputations due to infection (Kementerian Kesehatan RI, 2014; Richard, 2011; Shaw et al., 2010).

It is important to prevent the progression of diabetic foot ulcers which require proper wound care. Therefore, wound dressing selection in diabetic foot ulcer patients will determine the outcome and effectiveness of the wound healing process. An ideal dressing should be nontoxic, hypoallergenic, protect against bacterial infection, comfortable, can maintain moisture, easy to remove, and inexpensive (Dhivya et al., 2015; Richard, 2011). Hydrogel film can facilitate a humid environment in the area of injury, is flexible, soft, and absorb water so that it is appropriate to be used in festering wounds or discharge such as diabetic ulcers (Dhivya et al., 2015). One of the most widely used hydrogel bases is chitosan. Chitosan is non-toxic and does not trigger an immune response, biodegradable and also reported to have antimicrobial activity, especially against the bacterium *Staphylococcus aureus* which commonly infects diabetes wounds (Escárcega-Galaz et al., 2018; Kavitha, 2014; Rabea et al., 2003). In addition, chitosan is known to accelerate blood clotting. Therefore, chitosan-based hydrogels film not only function as drug carriers but also effective wound dressing (Hussain et al., 2018).

Vitex trifolia L or Legundi is reported to have anti-inflammatory activity, antibacterial activity against gram-positive and negative, and antioxidant activity (with IC₅₀ 40.0-226.7 µg/mL) (Kulkarni, 2011; Aweng et al., 2012; Hossain et al., 2001; Kannathasan et al., 2011; Matsui et al., 2009). The flavonoid compound, vitexcarpin, shows significant inhibitory potential for the proliferation of T lymphocytes, B cells, and inflammatory mediators such as cytokines which promote the wound healing process (Matsui et al., 2009). Legundi leaf extract gel (5% (w/w)) is also reported to have good wound healing ability for incision wounds, excision, and dead wound space (Shaw et al., 2010). The combination of chitosan as a carrier and Legundi leaf extract which both have anti-inflammatory, antioxidant, and antibacterial activities will increase its effectiveness as a wound healing agent. Therefore, it is necessary to perform further research to evaluate the effect of chitosan-based hydrogel film loaded with leaf extract of Legundi (*Vitex trifolia* L.) on the wound healing process under hyperglycemic conditions. This study was conducted to optimize the concentration of chitosan, PVP K30, and PVA towards the Legundi extract film properties, and to evaluate its wound healing activity on hyperglycemic male BALB/c mice using an incision wound model.

MATERIALS AND METHOD

Materials

Plant materials and preparation of plant extract

The leaves of *Vitex trifolia* L. or Legundi (LGD) were collected from a rural area of Denpasar, Bali. Chemicals used in this study were alloxan monohydrate (Sigma, St. Louis, MO, USA), normal saline (Otsuka, Indonesia), ketamine/xylazine, DPPH, ascorbic acid, chitosan (Biotech Surindo), polyvinylpyrrolidone (PVP K-30), polyvinylalcohol (PVA), propylene glycol, demineralized water, and ethanol.

Male BALB/c mice (6-8 weeks old) were purchased from the animal unit laboratory, section of the Pharmacology Department, Medicine Faculty, Udayana University. All mice were housed in an air conditional animal house at a controlled room temperature (22–24°C) with a 12 h natural light/dark cycle. Water and a standard chow diet were provided ad libitum. All animals were acclimatized for two weeks before the experiment. Animal experiments and care procedures were

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approved by the Research Ethics Committee, Veterinary Medicine Faculty, Udayana University (no. 2856/UN14.2.9/PD/2019 on September 2019).

Methods

Plant extraction

The leaves were collected, then washed, dried, and powdered. Two hundred and fifty grams of coarse dried leaf powder was later extracted using one liter of ethanol 70% by maceration method for 3 days. The extract was then filtered and concentrated in a rotary evaporator at 40°C to obtain the semisolid-like materials. The viscous residue was kept in the oven at 30°C until a dry solid mass was obtained (yield 9.6%). The extract is then kept in the refrigerator until further use.

Flavonoid and total phenolic content of plant extract

The flavonoid content of LGD ethanolic leaf extract was determined using a colorimetric assay by aluminum complex formation using quercetin as standard, the amount of flavonoid content was expressed by milligram of quercetin equivalent per 100gram extract. The total phenolic content of LGD extract was determined by Folin-Ciocalteu reagent using gallic acid standard and expressed by milligram of gallic acid equivalent (GAE) per 100gram extract. All of the assays were performed in triplicate by the Integrated Laboratory Unit, Agriculture Faculty, Udayana University.

Antioxidant activity (DPPH scavenging assay) of plant extract

The ability of LGD extract to scavenge 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radicals was carried out by dissolving 100 mg extract in ethanol to make 1 mg/mL stock solution. Serial dilution of extract (100; 125; 150; 175; 200 ppm) of each extract was made by dilution using ethanol from stock. To 200 µL from each dilution series were added 1.8 mL ethanol and 2 mL DPPH 0.2 mM solution then the mixture was incubated at room temperature for 30 min. the control consisted of 2 mL DPPH solution and 2 mL of ethanol. Afterward, the absorbance was measured at 517 nm in Genesys™ 10S UV-Vis spectrophotometer. Ascorbic acid was used as standard. The percentage of inhibition of the DPPH free radical was calculated through the following equation:

$$\% \text{ inhibition} = 100 - \frac{A_{\text{sample}}}{A_{\text{control}} \times 100} \quad (1)$$

Calculation of IC₅₀ of each LGD extract and ascorbic acid was done by plotting the % inhibition against sample concentration and the linear regression analysis and used to indicate antioxidant capacity.

Experimental design for Vitex trifolia L. extract (LGD) loaded chitosan hydrogel film

A simplex lattice design was applied for LGD extract-loaded chitosan-based film formulation to evaluate the influence of film composition (Chitosan; PVA: PVP K30) on physical characteristics of the resulted film such as thickness, weight uniformity, folding endurance, swelling index, and the swelling rate in two different levels. The significant effect of these independent variables in Table 1, was investigated on the dependent variables using Design-Expert® 10.0.6.0 software. The design generated 14 runs as shown in Table 2. The composition of chitosan, PVA, and PVP K30 in the optimized formula was determined using numerical optimization. In this method, a goal was set for each response whether the optimal condition would be in range, maximize, minimize, equal to a certain value, or targeted. Then the solution was calculated to generate optimal conditions, and the optimal formula was chosen based on the desirability value.

Table 1. Factor and level in the simplex lattice design (SLD)

Factor	Level	
	Low	High
Chitosan (%b/v)	1	1.5
PVP K-30 (%b/v)	1.25	1.75
PVA (%b/v)	1.25	1.75

Preparation of LGD extract-loaded chitosan hydrogel film

Hydrogel film was prepared using the solvent casting method in different concentrations of chitosan, PVP-K30, and PVA as given in Table 2. Chitosan solution was prepared by dissolving to 1.5% w/v acetic acid glacial in demineralized water with occasional stirring for 2 hours (800 rpm, T= 30°C). The required amount of PVP K-30 was then dissolved into the chitosan solution. PVA was added to a suitable volume of demineralized water, then heated in a separated flask and stirred vigorously until all PVA powder was dissolved. PVA solution then was added to chitosan and PVP pre-mixture. This base matrix solution was made into 40 mL for each run. About 636.2 mg *Vitex trifolia* L. or Legundi leaf extract (LGD) was dissolved in 7.5 mL ethanol 70%. The dissolved extract and 2.5 mL propylene glycol (PG) were then added to the matrix base and stirred for 1 hour. This solution was left to rest to remove air bubbles. Finally, the respective LGD-film-based solution (50 mL) was cast over the petri dishes (diameters of 9 cm) and the solvent was evaporated by oven at 30°C for 48 hours (BINDER ED 115, Germany). Dried LGD extract-loaded hydrogel films were carefully peeled and stored in a desiccator for further experiments.

Film physical properties determination

Thickness and weight uniformity

The thickness of three randomly selected films from every run was determined using a digital caliper gauge. Weight uniformity of film was determined by taking the weight of ten films of sizes 1 cm² from every run and weighing them individually on an electronic balance.

Folding endurance

The folding endurance of the film was determined by repeatedly folding one film at the same place till it broke or folded up to 100 times manually, which was considered satisfactory to reveal good patch properties. This test was done on randomly selected three films from each run.

Swelling index and rate study

The films cut into 1 cm² were weighted accurately and kept immersed in 5 mL of demineralized water. The films were taken out carefully at minutes 1, 3, 5, 10, 15, 30, and 45 then blotted with filter paper to remove the water on their surface and weighted accurately (Kavitha, 2014). The swelling index was calculated using equation 2.

$$\% S = (X_t - X_o / X_o) \times 100 \quad (2)$$

Where, X_t – is the weight or area of the swollen patch after time t and X_o - is the original patch weight or area at zero time. Higuchi kinetic model was used to determine the swelling rate of the film by plotting the swelling index against time intervals. The swelling rate (kH) was expressed as the slope of the line regression of the plot.

Wound healing activity of LGD extract loaded chitosan hydrogel film on hyperglycemic mice hyperglycemia Induction in mice

All mice were weighed and checked for fasting blood glucose levels using GlucoDr AGM 2100 (Korea) before induction. Animals showing very low or high glucose levels were excluded. Mice were made hyperglycemia with a single intraperitoneal injection of alloxan monohydrate (200

mg/kg BW) in saline (154 mM NaCl). Two days after the alloxan injection, mice with fasting blood glucose levels of >140 mg/dl were included in the study. Treatment with LGD extract-loaded chitosan hydrogel film was started 24h after alloxan injection. For blood glucose measurement, the blood was drawn from the tail vein.

Incision wound model

Animals were anesthetized by intraperitoneal injection of ketamine-xylazine combination (K 100 mg./kg BW; X 10 mg/kg BW), and the back hairs of the mice were shaved 4 cm x 2.5 cm to facilitate the incision process. The incision wound was made using a scalpel (length of \pm 1 cm; depth of \pm 5 mm). Animals were then divided into three groups with 9 mice in each group, which receive the following treatment:

- Group I: Control group (Mice received alloxan injection and wounds were cleaned from intact debris using normal saline)
- Group II: Placebo group (Mice received alloxan injection and wounds were treated by applying 1x 1 cm placebo film once daily)
- Group III: LGD Film group (Mice received alloxan injection and wound treated by applying 1x 1 cm LGD extract Chitosan Based Film group once daily)

Wound lengths were measured on days 0, 1, 4, and 8 using a digital caliper. Wound healing was evaluated using parameters of the percentage and time of wound closure. The percentage of wound closure was determined by using equation 3.

$$((L1 - L2) / L1) \times 100 \quad (3)$$

Where, L1 is the length of the wound on day 0, while L2 is the length of the wound on the day of observation. All data were then analyzed by one-way ANOVA using SPSS version 16 (IBM™).

RESULT AND DISCUSSION

Flavonoid and total phenolic content of LGD extract

The flavonoid and total phenolic content of LGD extract were found to be 497.16mg/100g equivalent of quercetin (QE) and 1753.2 mg/100g equivalent of gallic acid. This shows that *Vitex trifolia* L or Legundi (LGD) leaf extract had proven to be a good source of flavonoids and polyphenolics. However, the phytochemicals content of LGD obtained in the Denpasar area was lower in both flavonoid and total phenolic compared to the result given by (Saklani et al., 2017) which were found to be 57.41mg/g QE and 77.20mg/g GAE respectively.

Antioxidant activity (DPPH scavenging assay) of LGD extract

To evaluate the antioxidant activity of LGD leaf extract, DPPH free radical scavenging activity was used and compared against control such as ascorbic acid. Crude ethanolic extract of Legundi was found to have a potential antioxidant activity (IC_{50} = 56.68 ppm), whereas, the IC_{50} of ascorbic acid was 6.44 ppm. A lower IC_{50} indicates higher antioxidant properties, which suggests that LGD extract has lower (10 folds lower) antioxidant activity than ascorbic acid (Saklani et al., 2017).

Optimization of LGD leaf extract chitosan-based hydrogel film

The film composition of each experimental run generated by Design-Expert® software were presented in Table 2. Three-component interactions were adopted under Simplex Lattice Design to assess all main effects, as well as to determine the component and interaction points that influence the responses (i.e thickness, weight, folding endurance, swelling index, and swelling rates based on Higuchi equation (kH)). Table 2 includes the value of (Mean \pm SD) of weights, thickness, folding endurance, swelling index, and swelling rate (kH). The best fit models generated by the software

(Design Expert 10®) for the observed responses shown in Table 3 included a quadratic model for film thickness and weight, a special cubic model for film's folding endurance and swelling rate (kH), and a linear model for swelling index. Apart from the equations provided by the software, a contour plot from each response was also provided as seen in Figure 1. The contour plot is a two-dimensional (2D) representation of the response plotted against combinations of numeric factors and/or mixture components. It can show the relationship between the responses, mixture components, and/or numeric factors, thus facilitating the visual interpretation of the results (Aminu et al., 2018). Lack of fit in Table 3 described the fitness of the model in determining the interaction between responses and factors, a not significant value of lack of fit is needed to model to fit and the response is used in optimization (Nazmi & Sarbon, 2019). The lack of fit value on all responses was found to be not significant.

Table 2. SLD experimental design runs for LGD extract chitosan-based film, film composition, and physical characterization result

Code	Film composition			Characteristics						
	Factor	PG (mL)	LGD (mg)	Thickness (mm) n=3	Weight (mg) n=10	Folding endurance n=3	Swelling index n=1	Swelling rate/kH (mg/min) n=1		
	Chitosan (% b/v)	PVP K30 (% b/v)	PVA (%) b/v)							
Run 1	1.25	1.25	1.5	2.5	636.2	0.38	70.22	225	0.71	11.33
Run 2	1.25	1.5	1.25	2.5	636.2	0.67	75.43	200	0.63	10.99
Run 3	1.33	1.33	1.33	2.5	636.2	0.47	81.56	33	0.70	11.76
Run 4	1	1.25	1.75	2.5	636.2	0.52	64.36	135	0.83	11.66
Run 5	1	1.5	1.5	2.5	636.2	0.46	69.23	289	0.72	11.26
Run 6	1.08	1.58	1.33	2.5	636.2	0.60	65.24	100	0.72	11.68
Run 7	1.17	1.42	1.42	2.5	636.2	0.53	70.44	67	0.85	13.80
Run 8	1.5	1.25	1.25	2.5	636.2	0.66	85.98	56	0.36	7.53
Run 9	1.08	1.33	1.58	2.5	636.2	0.46	67.4	33	0.61	12.48
Run 10	1	1.75	1.25	2.5	636.2	0.48	60.58	50	0.61	10.48
Run 11	1	1.75	1.25	2.5	636.2	0.64	62.28	69	0.70	11.10
Run 12	1.25	1.5	1.25	2.5	636.2	0.58	74.89	123	0.50	8.91
Run 13	1	1.25	1.75	2.5	636.2	0.50	67.31	198	0.65	9.91
Run 14	1.5	1.25	1.25	2.5	636.2	0.63	83.51	25	0.42	7.91

Thickness and weight uniformity of experimental runs

The film of LGD extract which made of different concentrations of chitosan, PVP K30, and PVA was ranged from 0.38 to 0.67 mm. The quadratic equation in Table 3 described the influence of the film composition (chitosan, PVP K30, and PVA) on the film thickness which showed that the interaction of chitosan and PVA (AC) has more influence to increase film thickness as it has the highest and positive coefficient value. Chitosan and PVA have a higher viscosity than PVP K-30, therefore the combination of both will increase the film thickness. PVA also acts as a bridge to more than one chitosan molecule, which shortens the distance between each chitosan molecule. Thus, creating a more compact film which increases the film's thickness and density. As shown in Table 2 average weight of film from each run ranges from 60.58 to 85.98 mg. Based on the quadratic equation in Table 3, chitosan (A) contributed most of the film weight. This is because chitosan has a higher

molecular weight and viscosity compared to the other polymers (PVA and PVP K30). Therefore, the increase of chitosan concentration in the film will increase the weight of the film.

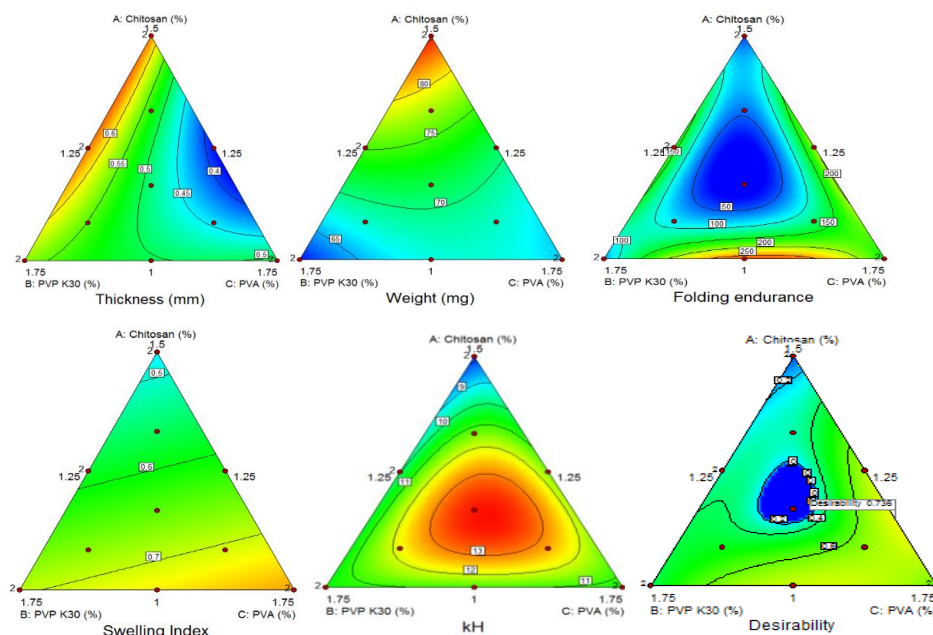


Figure 1. Response surface plots for the design indicate the effect of formulation factors on the responses obtained using actual components. This color in the contour plot is based on response value. The red area indicated the higher value of the responses while the blue shows the lowest

Folding endurance

A good wound dressing film should retain its integrity from folding because it needs to maintain flexibility while used on the skin (Poonguzhali et al., 2018). Based on Table 2, the folding endurance of the film in each run is very varied, which ranged from 25 to 289. To explain the influence of film components on folding endurance, the special cubic model was used. Table 3 and Figure 1 showed that PVA was a dominant variable that affected the folding endurance of the film as the higher the ratio of PVA, the higher the flexibility of the film (shown as the red area of the contour plot). Chitosan is a linear polysaccharide formed by the copolymerization of d-glucosamine and N-acetyl-d-glucosamine, while PVA is often an atactic polymer. The rigid structure of d-glucosamine and N-acetyl-d-glucosamine make chitosan more difficult to rotate and less flexible than linear PVA, hence the tensile strength of PVA films is larger than CS (Vo et al., 2019). The addition of PVA into chitosan film which contains a hydroxyl group will form hydrogen bonding and an electrostatic bond with some polycations such as chitosan. This will prolong the entanglement among chitosan/PVP K30/PVA blend, which led to the formation of difficult-to-break film and possession of higher folding endurance, therefore PVA is the dominant factor in film's folding endurance (Vo et al., 2019).

Swelling index and rate study

Based on Table 2 and Figure 1, the swelling index tends to increase with increasing concentration of PVA which follows a linear model. However, all the film components showed a positive coefficient value in the linear model, which means not only PVA but both chitosan and PVP K30 also contributed to increasing the swelling index. The swelling index could be used as an indicator of the water absorptivity of the film. Therefore, the extent of cross-linkage of the polymer

blend in the films would improve the mechanical properties and the water resistance of the films which led to a lower swelling index (Vo et al., 2019). It is desirable for a wound dressing to have good release properties of the drug from the matrix, to facilitate the healing process. The release of the hydrogel film was influenced by the swelling index and swelling rate of the film. Both PVA and PVP K3, are water-soluble polymers, therefore the addition of these polymers into chitosan film will increase its swelling ability and rate (Poonguzhali et al., 2018; Vo et al., 2019).

Table 2 also showed that the swelling kinetics of the films made from the chitosan/PVPK30/PVA blend followed Higuchi's diffusion equation. Higuchi model indicates that drug diffuses through pores in the polymer matrix (Notario-Pérez et al., 2017). A hydrophilic polymer such as chitosan does not disintegrate completely when in contact with an aqueous medium but undergoes moderate swelling due to the relaxation of the polymer chains (Notario-Pérez et al., 2017). However, the low water solubility of chitosan decreases the swelling process compared to other hydrophilic polymers (Kang et al., 2018), whereas, PVA and PVP K30 has undergone erosion due to their higher solubility in water. Therefore, when chitosan film contact with water, the absorbed water inside the film will solubilize PVA and PVP K30 and create pores inside the chitosan matrix, thus lead the later swelling mechanism following Higuchi model.

Table 3. Determination of mixture model and final equation of parameter against actual film component

Optimization Parameters (Y)	Mixture model	The final equation in the actual component	Lack of fit
Thickness	Quadratic (p=0.0293)	Y=2.31A-0.72B+2.7C+0.572AB-2.97AC-0.53BC	not significant (p = 0.664)
Weight	Quadratic (p= <0.001)	Y= 115.95A-103.3B+12.06C+31.39AC-74.47AC+68.06BC	Not significant (p = 0.123)
Folding endurance	Special cubic (p=0.0420)	Y = -63235.24A-47732.43B+ 7349.67C+66848.57AB+66644.05AC+44644.46BC-52025.79ABC	Not significant (p = 0.1341)
Swelling index	Linear (p=0.0248)	Y=-0.21A+0.24B+0.38896C	Not significant (p = 0.286)
Swelling rate (higuchi/kH)	Special cubic (p=0.0057)	Y=590.59A+467.02B+443.84C-664.11AB-640.84AC-535.07BC+540.23ABC	Not significant (p = 0.897)

A= Chitosan; B= PVP K30; C= PVA. The coefficient of each variable shows its degree of contributive effect on the responses, while the plus or minus sign signifies its boosting or castrating impact.

Numerical optimization of Chitosan/PVP K30/PVA in LGD films

Table 4. Predicted vs actual responses obtained for the optimized formulation

Responses*	Thickness (mm)	Weight (mg)	Folding endurance	Swelling index	Swelling rate (kH)
Actual values (n=3)	0.42±0.06	67.54±1.89	204.67±2.52	0.62±0.04	10.22±0.34
Predicted values	0.39	67.6	203.4	0.684	11.67
p-value (sig<0.05)	0.405	0.963	0.475	0.107	0.018

*Formula optimum consisted of 1.15 % Chitosan, 1.25% PVP K30, and 1.6 % PVA, with a desirability of 0.736

Based on the ideal criteria of film as wound dressing, the optimal formula of LGD leaf extracts chitosan hydrogel film should be thin, lightweight, flexible, and have a moderate swelling index and rate to remove wound exudate and promotes the healing process. Therefore, to optimize the composition of chitosan/PVP K30/PVA in thickness and weight responses goals were set to minimize, while the folding endurance, swelling index, and rates goals were set to maximize. Based on this criterion, the software generates 3 formulas. The formula with the highest desirability value

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(0.736) which consisted of 1.15 % b/v chitosan, 1.25% b/v of PVP K30, and 1.6% b/v of PVA was chosen as the optimum formula. Replicates of optimum formula and placebo films were prepared according to this composition and were characterized by the same parameters as the runs, then used in in vivo study to observe its wound healing activity. Based on Table 4, there was an acceptable agreement between predicted versus observed values for the optimized formulations on all responses except the swelling rate ($p < 0.05$). However, this still showed the reliability of the used optimization model.

Wound healing activity of LGD leaf extract chitosan hydrogel film in hyperglycemic mice

Table 5. Effect of LGD leaf extract chitosan hydrogel film on wound closure of hyperglycemic mice at a different time interval

Group	Wound closure (%) [*]		
	Day 1	Day 4	Day 8
Group I (A)	5.11±5.03	33.90±5.05	69.27±1.93
Group II (B)	9.54±6.93	38.21±4.18	75.88±5.61 ^a
Group III (C)	9.98±7.77	50.70±7.97 ^{a,b}	85.48±6.63 ^{a,b}

^{*} Data represent the means of 9 replications, ^a = $p < 0.05$ compared to group I; ^b = $p < 0.05$ compared to group II

The wound healing activity of LGD films was expressed by the wound closure percentage after the application of film on the wound surface at a different time interval which can be seen in Table 5 and Figure 2. Macroscopically, the wound dressed with LGD films showed considerable signs of dermal healing and significantly heal faster compared to the placebo group and control group as seen in Figure 2. A similar result was found in statistical analysis of wound closure percentage which showed that, after day 4 and day 8, mice treated with LGD films have higher and significantly different wound closure percentages ($p < 0.05$). Placebo film also has the potential activity to accelerate healing because the wound closure percentage in the placebo group was significantly higher on day 8 compared to the control group. Though there was no LGD extract in the placebo, chitosan as a drug vehicle on this dosage form has been known to promote thrombosis and blood coagulation by affecting platelets activation and blocking nerve ending, which reduces pain and increases patient compliance. The chitosan-based hydrogel also helps regulate the activity and release of inflammatory mediators and cells, thus creating a suitable condition for healing (Liu et al., 2018; Thangavel et al., 2017). Upon contact with the wound surface, chitosan will degrade gradually and release *N*-acetyl- β -d-glucosamine which has the same backbone structure as hyaluronic acid (HA). Thus, will increase natural HA levels, stimulate the proliferation of fibroblasts, and increase angiogenesis, and collagen deposition at the wound site which faster the healing process (Liu et al., 2018). The antibacterial properties of chitosan also help control infection in chronic wounds such as diabetic foot ulcers (Liu et al., 2018; Rabea et al., 2003). It has been proven that phytochemical constituents of *Vitex trifolia* L or Legundi such as catechins, flavonoids, and other flavonoids can significantly improve wound healing and scar formation because of their antioxidant, antibacterial, and antiinflammatory activities (Aweng et al., 2012; Kannathasan et al., 2011; Manjunatha et al., 2007; Matsui et al., 2009). Therefore, in LGD films, when chitosan is combined with ethanolic LGD leaf extract which contains phenolic and flavonoid and is a good antioxidant, the wound healing activity of the chitosan-based film will increase.

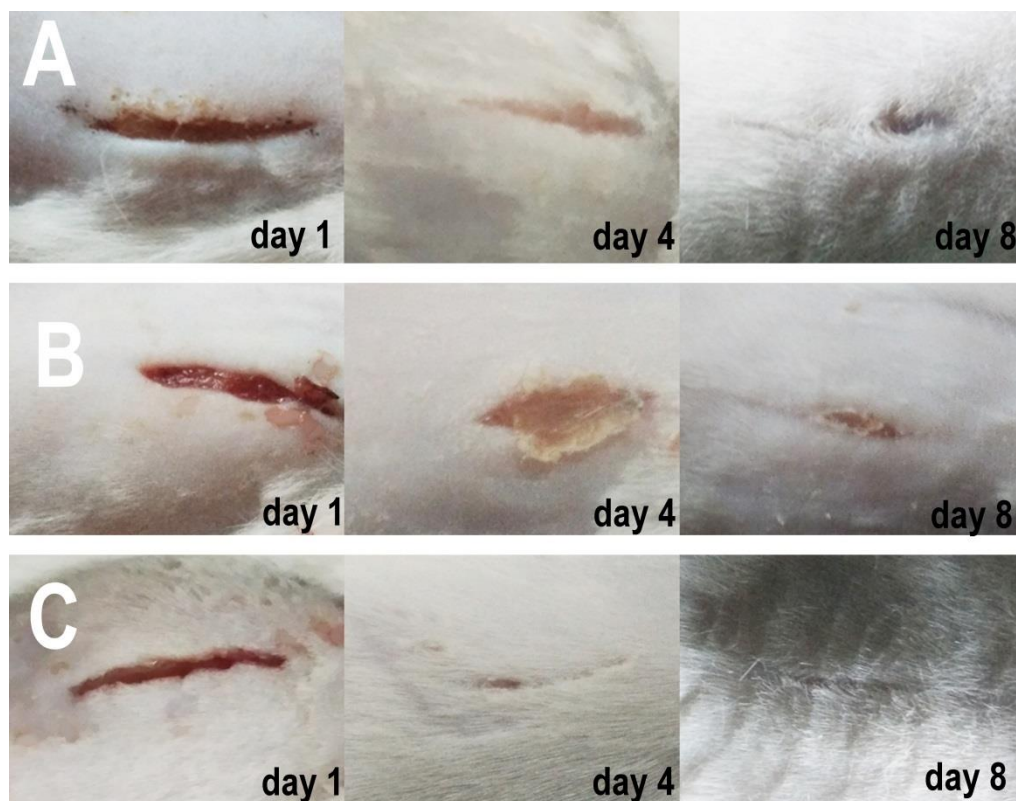


Figure 2. Photographs of macroscopic wound healing observations at different time intervals in incision wound model in mice. A= group I (control group); B = group II (placebo group); C= group III (LGD film group). The wound size indicated that mice in group III healed faster compare to the group I and II

CONCLUSION

In this study, we have developed a chitosan-based hydrogel film loaded with *Vitex trifolia* L., or Legundi (LGD) leaf extract. The results suggested that chitosan and PVA were responsible for affecting the film's thickness, weight, and folding endurance. While PVP K30 was the dominant factor in increasing the swelling index and rate of the film. The optimum LGD films were consists of 1.15 %b/v chitosan, 1.25 %b/v of PVP K30, and 1.6 %b/v of PVA, and have good film characteristics such as being thin, lightweight, flexible (good folding endurance), good swelling index and rate yet still maintained its integrity throughout the assay. Wound treatment using LGD film effectively enhanced the healing process in hyperglycemic rats. Therefore, it can be concluded that the developed chitosan hydrogel film loaded with LGD leaf extract exhibits a promising wound healing activity and can thus serve as a potential alternative to wound dressing for the diabetic wound.

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