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MORINGA OLEIFERA LEAF EXTRACT LOADED HYDROGEL FOR DIABETIC WOUND HEALING

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ABSTRACT

Diabetic wounds (DW) are a chronic, non-healing wound on the feet of diabetic patients that pose a serious challenge to world health. Around 84% of diabetic patients undergo lower leg amputations. Though numerous topical and systemic drugs have been used to heal the DW, these drugs have led to the emergence and subsequent rapid overgrowth of resistant bacterial strains, side effects and toxicity. Many herbal plants have very important role in wound healing because they promote the natural repair mechanisms. *Moringa oleifera* (MO) is an important medicinal plant which has an impressive range of medicinal uses including antimicrobial, anti-inflammatory, antidiabetic, antioxidant and anticancer activities.Recently few researchers reported that MO extracts have effective wound healing property due to the presence of rich flavonoids and vicenin-2.

The objective of the present study was to develop hydrogel formulations loaded with *Moringa oleifera* leaves extract. The prepared hydrogels were evaluated for physical appearance, rheological behavior, skin irritation and wound-healing power in streptozotocin-induced diabetic male wistar albino rats. Results showed that all hydrogel formulations exhibited good and acceptable physical properties. All the animals tolerated the applied gels and no signs of irritations were noticed during the skin irritation study. The *in-vivo* wound healing studies showed a time dependent increase in percentage of wound, a contraction which is higher than that produced by the control groups. These contractions were statistically significant (P<0.001), during the first 10 days of the study with MO-Hydrogel administration. The MO-hydrogel showed the highest percent wound contraction with complete wound closure and epithelization was observed on 7thday of wound induction.

Keywords: Wound healing efficacy, Moringa oleifera, Hydrogel, Diabetic wound, Streptozotocin.

INTRODUCTION

Diabetes and its complications are quickly becoming an epidemic in all around the world. As globalization is inevitable, so is the inheritance of the risks of non-communicable diseases which are overwhelming in developed countries.It is estimated that 2.6 million people are diabetic,which is 15% of the total population, and these numbers have been projected to increase sharply over the years. Diabetic foot disease is said to affect 15%to 25% of diabetics in the course of their lives and is the leading cause of non-traumatic amputations of the lower limb (Wild *et al*.,2004). This significant diabetic complication not only affects the patient's life, but their families and the country, which eventually leads to exorbitant treatment costs and hamper the growth of the country (Karakkattu *et al*., 2014).

Despite the fact that many reports have been published and various new drugs are currently undergoing clinical evaluation for the treatment of diabetic wounds, however, convincing set of evidences has not been established till date. Further, once a wound has formed, noninvasive therapies are less effective and invasive therapies are costly. Hence, a single treatment strategy (multi-mechanism-based products) to address all the pathological conditions of chronic diabetic wound needs to be developed (Brem & Tomic-Canic, 2007). Herbal medicines have an incredibly important role in wound healing because they promote the natural

mechanisms (Dorai, 2012). Plant extracts like flavonoids, terpenoids, tannins and xanthones, when administered through novel drug delivery system, show enhanced absorption which enables them to cross the biological membrane,which leads to greater bioavailability. *Moringa oleifera* (MO) is a significant medicinal plant which has an impressive range of medicinal uses including antimicrobial, anti-inflammatory, antidiabetic, antioxidant and anticancer activities. Recently, researchers reported that MO extracts have effective wound healing property because of the presence of rich flavonoids and vicenin-2 (Gothai *et al*., 2016). However, leaf extract as such to apply on the wound is not convenient and difficult to attain better therapeutic effect. Hence, there is urgency to revolve the wholesome benefits of herbal medicine into modern drug delivery system. It has synergistic potential to treat various chronic diseases with lowest overall cost of the therapy.

Carbopol based hydrogels drug delivery has been extensively studied for diabetic wound healing applications during the past decade for its exclusive advantages including fluid absorption and hydration of the wound bed (Zinov'ev,2014).Carbopol is made of carbomers.It is cross-linked together and makes a microgel structure that makes them optimal to be used as a drug delivery carrier for dermatological applications (Guo, 2003).The drug delivery can be used to achieve controlled release when desired. This microgel structure makes it possible for these systems to tolerate the physical movement of the body and shape themselves after application (Islam *et al*., 2004). It is cross-linked with polyalkenyl ethers or divinyl glycol. These polymers are anionic polymers that need naturalization to become gellified. Organic amines like triethanolamine can be used to naturalize these polymers in liquids. Carbopol Ultrez 20 is a new polymer with an excellent rate of dispersion and it forms gels without extensive dispersion (Guo, 2012).

Therefore, this study was aimed to develop Carbopol (Ultrez 20) based hydrogel system for MO extract to explore its wound healing potential. MO-CH has been formulated and investigated for its various formulation parameters. The wound healing efficacy was evaluated using streptozotocin induced diabetic albino rats.

MATERIALS AND METHODS

Carbopol (Ultrez 20), Streptozotocin from SRL chemicals, India. All other chemicals and reagents used for this research were analytical grade.

Plant collection and extracts preparation:

Matured leaves of *M. oleifera* were collected from the farm,Universiti Putra Malaysia, during the rainy season and authenticated by a botanist. A voucher specimen (SK 1561/10) was deposited at the herbarium of Institute of Bioscience. The air-dried and powdered leaves were extracted by a maceration process with 1:1 ratio of methanol and distilled water with the help of shaking the incubator set at 27°C for 24 hours. Extracts were then filtered and evaporated to dryness under reduced pressure with rotary evaporator. The dried crude extracts were stored in a refrigerator for further use.

Formulation of M. Hydrogel:

Carbopol (Ultrez 20) has very good dispersion ability and forms gels rapidly. In brief, the gels (0.2, 0.5 and 1% w/w, respectively) were prepared by thefollowing procedure (Skalko *et al*., 1998). Carbopol resin (weight in grams) was dispersed in distilled water (volume according to the desired concentration of gel). The mixture was stirred until thickening occurred and then neutralized by drop-wise addition of 50% (w/w) triethanolamine, until a transparent gel appeared. Quantity of triethanolamine was adjusted to achieve gel with desired pH. The formulated MO-hydrogels were stored atroom temperature for 24 hours to stabilize.

Physical Characterization:

The prepared gels were inspected visually for their color and homogeneity.The spreadability of the gel formulations was determined by measuring the spreading diameter of 1g of gel between two horizontal plates (20 cm × 20 cm) after one minute.The standardized weight tied on the upper plate was 125 g. The results obtained were average of three determinations.The pH was measured, at room temperature, in each gel sample using digital pH meter which was calibrated before each use with standard buffer solutions.The pH of the gel formulations was performed at 1, 10, 45 and 60 days after preparation to detect any pH changes with time.

Viscosity Measurement:

Viscometer (Brookfield digital viscometer DV-III,

temperature (25 ±1°C) before the measurements were taken.

Microbial load test by disc diffusion method:

The formulations were dissolved in methanol and filtrated through 0.45 µm Millipore filters. Microbial load was then carried out by disc diffusion method using 100 µL of suspension containing 104 CFU/mL of bacteria, 110 CFU/mL of yeast and 122 spore/mL of fungi spread on NA,SDA and PDA medium, respectively. The blank discs were impregnated with 20 µL of dissolved compounds and placed on the inoculated agar. Negative controls were prepared using same volume of methanol employed to dissolve the synthetic compounds.Ofloxacin (5µg/disc) and nystatin (100 µl/disc) were used as positive reference standards for bacteria and fungi, respectively, to determine the sensitivity of one strain/isolate in each microbial species tested. The inoculated plates were incubated at 36±1˚C for 24 h for bacterial strains and 48 h for yeast, at 27±1˚C for 72 h for fungi isolates.Antimicrobial activity was evaluated by measuring the zone of inhibition against the test organisms (Öğütçü *et al*., 2008).

Antimicrobial Activity:

The anti-microbial activity of each formulation was assessed by cup plate method. The zone of inhibition was *measured in nutrient agar medium, employing Bacillus subtilis, Staphylococcus aureus, Escherichia coli* and *Pseudomonas aeruginosa* as test organisms (Patel *et al*., 2011).

Skin irritation studies:

Wistar rats (200-250 g) of either sex were used for testing of skin irritation. The animals were maintained on standard animal feed and had free access to water. The animals were kept under standard conditions. Hair was shaved from back and area of 4cm was marked on both sides, one side served as control while the other side was test.MO-hydrogel was applied (500 mg/animal) twice a day for 7 days and the site was observed for any sensitivity and reaction if any. It was graded as 0, 1, 2, 3 for no reaction, slight patchy erythema, slight but cofluent or moderate but patchy erythema and severe erythema with or without edema, respectively.

Stability testing of hydrogels:

Accelerated stability testing was used to study the influence of temperature on the gel stability. For that purpose, 0.5% Carbopol hydrogel with pH values of 4.2, 4.7, 5.8, 6.9, 8.2 and 10.4, respectively were kept in a thermostat at 40°C. After one-month period, the gels were analyzed on viscosity, pH and microbial content as previously described. w.

In vivo wound healing efficacy of MO-Hydrogel:

Induction of diabetes in animals

Healthy male Wistar rats weighing between 200- 250 g were used for the study. They were housed in polycarbonated cages with sterilized rice husk bedding under controlled condition of 12 hourslight/darkness cycle and a temperature of 25±2°C. The animals were fed with commercial rat feed and tap water and allowed to acclimatize to environmental condition for 7 days prior to the commencement of the study. The experiment followed guidelines of Institutional Animal Care Use Committee of Faculty of Pharmacy, Lincoln University College, Malaysia (Approval No: LUC/IAEC/2016/012).

Induction of hyperglycemic condition was done using a combination of STZ and NAD according to the dosage reported in previous studies. The animals were injected with 150 mg/kg of NAD via intraperitoneal route and inject 65 mg/kg of STZ after 15 minutes. The induction procedure was repeated after 24 hours with the same dose of NAD and STZ. Blood was drawn from the tail after 72 hours to ensure successful induction of hyperglycemia, and glucose level was measured using a commercial glucometer (Accu-Check glucometer, Hoffman-La Roche Ltd., Basel, Switzerland). Rats with elevated blood glucose level (≥10 mmol/L) were considered diabetic.Physical parameters such as body weight, food, and fluid intake were also recorded at specific intervals during the experimental period.

Induction of wound in diabetic animals and treatment

Prior to commencement of wound infliction, rats were anesthetized intramuscularly using xylazine 10 mg/kg and ketamine 90 mg/kg. The animals were shaved with an electric clipper at the dorsal back. Full thickness (6 mm diameter and 2 mm depth) of wounds were created using a sterile 6 mm biopsy punch. Wounds were left open and treated with prepared formulation.Various concentrations of formulated Hydrogel loaded *M. oleifera leaves* extract were applied topically to treat the animals twice daily for 21 days. The grouping of the

animals is shown in Table1.Wounds were photographed with a digital camera on day 0, 3, 7, 14, and 21 to assess the progress of wound closure. Wound size (surface area of the wound) was measured by tracing the wound on day 3, 7, 14, and 21 using a transparent paper and graph paper (Teoh *et al*.,2009).The percentage wound contraction (%WC) was also determined using a formula:

% WC= Initial wound size − wound size at specific day/ Initial wound size×100.

Table 1: Animal grouping and treatment

Group	Number of animals	Treatment	Dose
1	6	Vehicle	Normal control
2	6	Vehicle	Diabetic control
3	6	Formulation 1	0.5%
4	6	Formulation 2	1.0%
5	6	Formulation 3	2.0%
6	6		Market sample

Statistical analysis:

All values are reported as mean ± S.E.M. The statistical differences among groups were assessed using the Duncan multiple range test and analysis of variance (ANOVA).A value of $p<0.05$ was considered significant. Statistical analysis was performed using the SAS for Windows software.

RESULTS AND DISCUSSION

The Preliminary Phytochemical Analysis:

The preliminary phytochemical analysis of the hydroalcoholic extract of *M.oleifera* indicated the presence of saponins, carbohydrates, and alkaloids.

Physical Examination:

The physical properties of the gel formulations are shown in Table 2 and Figure 1.All gel formulations showed good homogeneity and spreadability. The physical appearance of formulations was transparent to pale green translucent nature according to the concentration of MO extract. The pH of the gel formulations was in the range of 5.8 ± 0.03 to 6.2 ± 0.23, which lies in the normal pH range of the skin and would not produce any skin irritation. It is important to mention that there was no significant change in the pH values as a function of time for all formulations. The viscosity values of all the prepared formulations were considered acceptable with the formulations F3 and F4,which were less viscous than the other formulations.

Table 2: Physical characterization of MO-Hydrogel

Formulation	Color	Appearance	Homogeneity	pH	Viscosity (cps)	Spreading efficiency (Diameter in mm)
F1	Colorless,	Thick; Transparent	Homogenous	5.6±0.03	8950±39	62±0.52
F2	Pale green color	Thick; Transparent	Homogenous	5.8±0.02	8870±49	68±0.39
F3	Pale green color	Thick; Translucent	Homogenous	5.8±0.05	9021±101	65±0.28
F4	Green color	Thick; Translucent	Homogenous	6.1±0.02	9215±59	71±0.47
F5	Green color	Thick; Translucent	Homogenous	6.2±0.01	9317±72	68±0.32

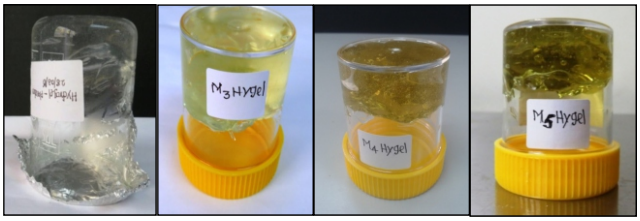

Figure 1: The digital photographic images of hydrogels (containing 0, 25, 50 and 100% of MO extract respectively) kept in inverted position.

Microbial load:

Microbial contaminants may be grouped into harmful, objectionable, and opportunistic organisms. Harmful organisms are toxin-producing, disease causing organisms such as *S. typhi, E. coli, P. aeruginosa, and S. aureus*. Objectionable organisms can cause disease or may interrupt the function of the agent leading to the deterioration of the product. These include *Salmonella spp*., (proteolytic types) and fungi (mycotoxin producing types), *Pseudomonas spp*., and *Candida albicans*. Organisms are said to be opportunistic if they produce disease or infection under special environmental conditions. Harmful organisms are excluded from all pharmaceutical products and excipients. With regard to the CPH pectin sample tested, no harmful microorganisms were identified (Table 3 and Figure 2) and the total microbial count was within the specified limit; hence, it passed the microbial quality test.

Table 3: Microbial load of MO extract and MO loaded hydrogels

Protocol	Results	Inference
MeOH extract with Nutrient agar	None detected	Passed
Hydrogel loaded MeOH extract with Nutrient agar	None detected	Passed
MeOH extract with Potato Dextrose Agar	None detected	Passed
Hydrogel loaded MeOH extract with Potato Dextrose Agar	None detected	Passed
MeOH extract with Sabouraud dextrose agar	None detected	Passed
Hydrogel loaded MeOH extract with Sabouraud dextrose agar	None detected	Passed

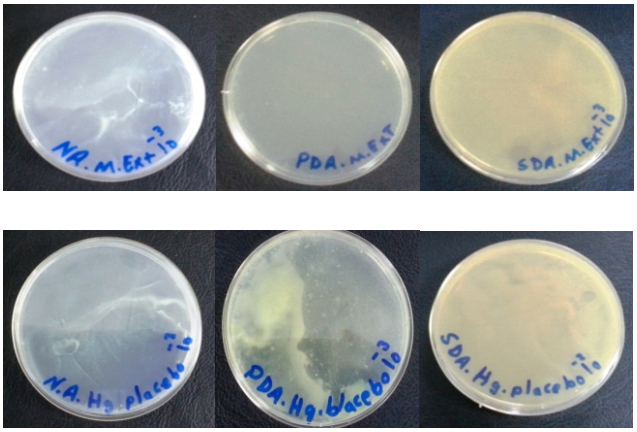

Figure 2: Microbial load of MO extract and MO loaded hydrogels

Antimicrobial activity:

Antimicrobial activity is one of the mechanisms by which some bioactive substances effect the wound healing. *Echerichia coli*, *Pseudomonasaeroginosa* and *Staphylococcus aureus* were selected as they comprise of the bacteria that commonly colonize on open wounds to cause poor healing. The benefits of antimicrobials in wound management have been established in numerous studies suggesting accelerated healing with either systemic or topical application of antibiotics; thus, endorsing antimicrobial activity as a mechanism of wound healing. In these studies, the concentration of MO extract does not have antibacterial effect against the selected organisms (Table 4 & Figure 3). These studies suggest that the pro-wound healing activity of Moringa oleifera leaves extract may causemechanism other than antibacterial activity.

Table 4: Antimicrobial efficacy of MO-Hydrogel

Test organism	Zone of inhibition (mm) of hydrogel			
	25%	50%	100%	Control
E. coli	Not found	Not found	Not found	Not found
Pseudomonas aeroginosa	Not found	Not found	Not found	Not found
Staphylococcus aureus	Not found	Not found	Not found	Not found

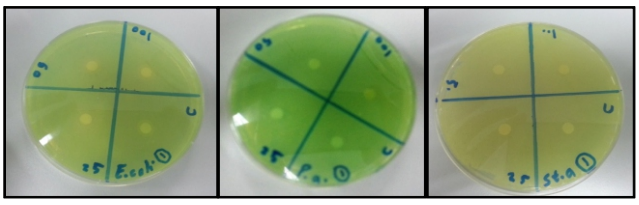

Figure 3: Antimicrobial activity of MO loaded hydrogel formulation against Echerichia coli, Pseudomonas aeroginosa and Staphylococcus aureus

Svkin Irritation Test:

All the animals tolerated the applied MO-hydrogels and no signs of irritations were detected during the whole period of study.

The measurement as presented in Figure 4 was done after the gel was tested for accelerated stability at 40°C (1-month period). As can be seen from Figure 4, the cohesiveness for almost all gels after they werestored at 40°C was found to be lower as compared to freshly prepared gel. The differences were more pronounced for pH values from 7 to 10.By increasing the temperature, the mobility of polymer chains increases, and we can see a fall in the gel cohesiveness. Increased mobility of polymer chains gives a shorter chain entanglement lifetime and it gives a more Newtonian flow to the gels (Fresno Contreras *et al.,* 2001)——. It is known from literature that PAA hydrogels do show changes in original structure after storage tests. This can be because of catalytic degradation of polymer chains and changes in the distribution of neutralizing ions within the gel. The ions within the gel can get released from the immediate icinity of the polymer gels into the bulk of aqueous phase (Tamburic *et al*.,1995).However, more recent publications on Carbopol Ultrez20 gels suggest a littleviscosity change under the normal storage and use of temperatures.One of the advantages of using Carbopol Ultrez 10 should be the fact that the possibility of unaccepted changes in the product's characteristic will be minimized.

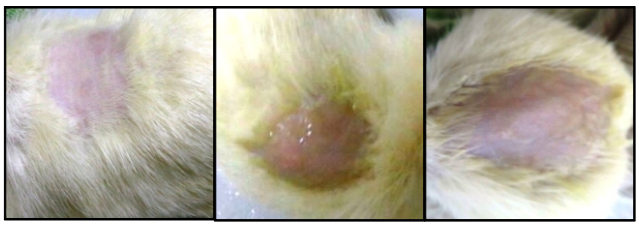

Figure 4: Skin irritation test (control, treated with MO-Hydrogel 0 hour and 4 hour)

Wound healing activity:

Wound contraction is another parameter used to assess wound healing. Significant wound healing activity was observed in animals treated with MO-Hydrogel in the diabetic wound. Wound contraction on day 8 in the extract treated group was 89.76% which was significantly (P<0.05) higher as compared to the control (45.75%) and standard (73.38%) groups. MO-Hydrogel

showed faster wound healing as compared to control group and wound contraction rate was also quicker (Figure5). This might be due to stimulation of interleukin-8,an inflammatory α-chemokine which affects the function and recruitment of various inflammatory cells, fibroblasts and keratinocytes (Moyer *et al*., 2002). On the basis of the result obtained in the present investigation, it is possible to conclude that the MO-hydrogel has significant wound healing activity.Several flavonoids including quercetin, kaempferol and isoquercetin present in MO leaves extracts are known to promote wound healing process.Based on the results, it is concluded that the *Moringa oleifera* leaves extract loaded hydrogel has significant wound healing activity in diabetes induced Wistar rats.

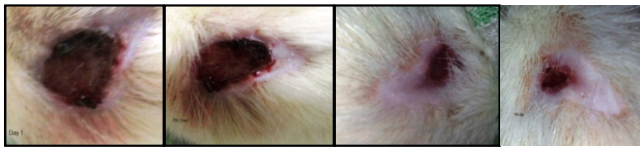

Figure5:Digital photographs of excision wounds induced diabetic induced animals with MO-Hydrogel treatment on day 1,7, 14, and 21.

CONCLUSION

Wound healing process consists of different phases such as granulation,collagenization,collagen maturation and scar maturation which are concurrent but independent to each other. Besides, new treatments are to be introduced (Eldor *et al*., 2004). However, most treatments are effective in mild to moderate wounds and the risk of amputation has not yet been addressed. Integration of the traditional herbal medicine with suitable drug delivery system is urgently needed for the treatment of diabetic wounds.The present study has demonstrated the promising wound healing activity of an aqueous fraction of *Moringa oleifera* leaves in an animal model of hyperglycemia through improved wound contraction, epithelialization, and modulation of inflammatory mediators. This confirms the in vitro wound healing activity earlier reported by our group.

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