



Honey incorporated antibacterial acellular dermal matrix for full-thickness wound healing

Archna Dhasmana^{1*}; Lubhan Singh²; Partha Roy³; Narayan Chandra Mishra¹

¹Department of Polymer & Process Engineering, Indian Institute of Technology Roorkee

²Department of Pharmacology, RIVET, Greater Noida

³Department of Biotechnology, Indian Institute of Technology Roorkee

*Corresponding Author(s): Archna Dhasmana

Department of Polymer & Process Engineering, Indian Institute of Technology Roorkee, India

Email: dhasmana15bio@gmail.com

Abstract

Honey is a potent natural antibacterial remedy used for the treatment of chronic wounds. In this study, Honey incorporated Acellular Dermal Matrix (H-AGDM) was fabricated for accelerating the wound healing process. The physiochemical and morphological characterization of the fabricated matrix was performed by FTIR, FESEM, *in vitro* degradation study, and antibacterial activity. Subsequently the biocompatibility was evaluated by *in vitro* 3T3 cell culture study and *in vivo* by full-thickness wound healing study in albino mice model. H-AGDM significantly showed significantly enhanced antibacterial activity, biocompatibility, anti-inflammatory response and wound healing rate.

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Introduction

Honey is a natural supersaturated sugar solution containing 80% *carbohydrates* which includes fructose, glucose, maltose, sucrose etc; ~17% water, and remaining 3% are protein, vitamin B etc [1,2].

Traditionally honey had been used as herbal medicine wound dressing material with multiple bioactivities, but scientific explanation of its wound healing mechanism was realized in the eighteenth century [3]. Honey has natural antibacterial and anti-inflammatory property, which promotes granulation tissue formation and high ECM (collagen and hydroxyproline) deposition [4,5]. The hyperosmotic effect (sugary content),

acidic pH, hydrogen peroxide (H₂O₂) production, and enzymatic action (glucose oxidase) of honey, provides bacterial resistance as well as inhibit the growth of antibiotic-resistant microbes (e.g., *S.aureus* and Methicillin-resistant staphylococcus aureus) [6,7]. During wound healing, honey slowly releases H₂O₂ which interacts with wound exudates, exhibits antibacterial property, and dilute concentration promotes cell proliferation and angiogenesis [1,8-10].

FDA approved honey for wound dressing applications and treatment of different types of wounds (e.g., burn, ulcers, and surgical wounds) [7,11-13]. Clinically, honey has been applied in the different forms, e.g., as ointment, hydrogel and honey



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impregnated in different dressing materials such as sterile gauze and bandages or a polyurethane dressing [14-16]. Preparing honey-impregnated dressing gauze is difficult and requires frequent replacement for it does not provide permanent wound-coverage [3]. Honey has been shown to increase the adherence of skin grafts to wound bed and increases the wound healing rate [13,17]. Therefore, many scientists fabricated honey modified polymeric (e.g., PVA, PEO, Chitosan and Silk) scaffold/matrix and used them as permanent skin substitutes for accelerated wound healing [16,18-21].

In this study, we developed a honey-based acellular graft for using as permanent wound-coverage and studied the synergistic effect of honey and acellular matrix on the acceleration of the wound healing process *in vivo*. Acellular grafts have porous 3D ECM (extracellular matrix) architecture consists of natural biomolecules, which provides biomimetic platform for repairing/regenerating damaged tissues and used as permanent wound-coverage [22].

For fabricating acellular skin-grafts, different types of cadaveric tissues (allogenic and xenogenic), e.g., SIS, Skin are used [23]. Allografts are biocompatible and non-immunogenic but they don't fulfil the demand for wound large area coverage. Besides, there are chances of infection, pain and morbidity at the donor site. Although, Xenografts (porcine and bovine tissues), overcomes the problems of tissue availability for covering large wound area but they have chances of disease transmission and evokes immune response [24]. Recently, many researchers focused on caprine tissue due to its less (or non) immunogenicity, biocompatibility and less susceptible to cattle disease (viruses and prions) transmission to human [25-27].

Therefore, combination of honey with acellular goat-dermal matrix (H-AGDM) forms a novel cost-effective honey based skin graft which is biocompatible, antibacterial, anti-inflammatory and provides an accelerated wound healing platform.

Experimental

Materials

For acellular dermal matrix fabrication, fresh cadaver goat-skin was brought from slaughterhouse. Raw Floral honey was purchased from Nature's Hut, Patiala, India. All the reagents/chemicals (nutrient medium, buffer, enzymes, antibiotics) were purchased from Himedia, India and Sigma Aldrich, India.

Preparation of matrix

Acellular matrix was fabricated using the procedure as explained by Mishra and co-workers [27]. Briefly, goat dermal tissue was subjected to decellularization under different physio-chemo enzymatic condition. For the effective removal of cells dermal tissue pieces were treated with 0.25% Trypsin-EDTA and 1% antibiotic (antimycotic) for 12 h at 25°C. Subsequently, the tissue sections were treated with 0.1 % SDS at 37°C for 6 h, followed by agitating in nuclease solution (RNase (20 µg/ml) and DNase (0.2 mg/ml) solution in 1:1 ratio) for 24 h at 37°C. After each treatment tissue samples were thoroughly washed by gently shaking with deionised water and 1X PBS. Finally, AGDM obtained was lyophilized and then sterilized with 70% ethanol for 30 min followed by UV ($\lambda=260$ nm) treatment for 4 h.

Subsequently, gamma radiation sterilized honey was lyophilized to obtain dry sample and prepared different concentration honey solution in deionised water. Sterilized AGDM of dimension 5 cm × 5 cm were dip coated in honey

solutions of concentration — 5%, 10%, 15% for 30 min and divided as sample 1, 2, and 3 respectively (Fig. 1). After incubation, samples (H-AGDM) were stored in deep-freezer at -40°C, lyophilized and characterized.

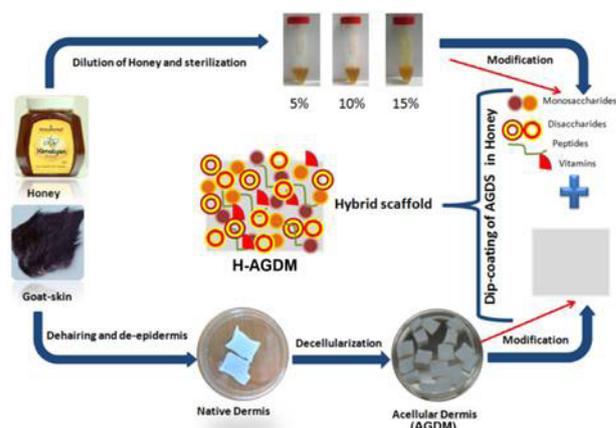


Figure 1: Schematic representation of fabrication of H-AGDM

FTIR analysis

For Fourier Transform Infrared Spectroscopy (FTIR) spectra analysis, freeze dried samples were mixed with KBr in ratio of 1mg sample per 900mg to form compressed pellets and the absorbance were recorded in the range from 4000 to 400 cm^{-1} at 2 cm^{-1} resolutions using FTIR spectrophotometer (Thermo Nicolet, USA).

Porosity

Porosity (χ) (n=5) of the matrixes were determined by the liquid displacement method [28] by following the equations as described below.

$$V_t = V_2 - V_3 \quad (1)$$

$$\chi = \frac{V_1 - V_3}{V_t} \times 100 \quad (2)$$

where, V_1 = initial volume of solvent, V_2 = volume of solvent after the sample immersion, V_3 = volume of solvent after removal of sample and V_t = total volume of the sample. All the experiments were repeated for 5 times ($n = 5$) and then the mean value was calculated.

Morphological analysis

Ultra-structure of the matrixes was determined using Field-Emission Scanning Electron Microscopy (FESEM) (MIRA3 TESCAN, Brno - Kohoutovice Czech Republic). The upper surface and cross-sectional area of matrix were examined at the saturation pressure of 1 torr and 5kV accelerating voltage. Further, the images were analyzed using ImageJ software (ImageJ 1.40 g, Wayne Rasband, National Institute of Health, USA).

In vitro biodegradation.

The biodegradability rate of the matrixes was evaluated under enzymatic as well as under non-enzymatic conditions [29]. Briefly, 5 mg lyophilized samples were separately immersed in 10 mL of 1X PBS enzymatic solution having 0.05% collagenase enzyme (125 U/mg) from *Clostridium histolyticum* and non-enzymatic 1X PBS (pH 7.4) respectively. All the samples

were incubated in a shaking incubator at 37°C and after regular time intervals of 5 h, samples were removed from the shaker, lyophilized and weighed. The rate of biodegradation ($n = 3$) was calculated by using the following equation:

$$\text{weight loss \%} = \frac{W_o - W_t}{W_o} \times 100 \quad (3)$$

where, W_o denotes the initial weight of the scaffold and W_t denotes the weight of the degraded sample at different time intervals.

Antibacterial activity

Antibacterial activity of the H-AGDM was evaluated by following the protocol as described earlier in the literature [30]. Briefly, bacterial suspension (*E. coli* and *S. aureus*) was prepared by inoculating the bacterial strain in MH broth and incubated them in a shaker at 37°C for overnight. Subsequently, 25 μ L enzymatically digested matrix was mixed separately in 100 μ L bacterial suspension (*E. coli* and *S. aureus*) and incubated the vials in a shaking incubator at 37°C. Bacterial suspension containing digested matrix was taken as test sample. Tetracycline (10 mg/mL in 50% methanol) was used as an antibiotic control for both *E. coli* and *S. aureus* bacterial cultures. *E. coli* and *S. aureus* bacterial suspension were taken as positive controls. 2 ml liquid culture medium from each group (control and test samples) was collected at different predetermined time intervals and measured the absorbance at 570 nm. Then, bacterial growth curve was plotted to study the antibacterial effect. In an effort to eliminate errors in the procedure, all assays were performed in triplicates ($n = 3$).

Biocompatibility study

The cell viability and proliferation over the matrix was studied by MTT assay with 3T3 mouse fibroblast cell line [31]. Briefly, the sterilized AGDM (control) and H-AGDM were incubated in DMEM nutrient medium for overnight in CO₂ incubator. After overnight incubation nutrient media was removed and 10 μ L of cell suspension (1×10^3 cells/well) medium was added over the wet matrix. Subsequently, freshly prepared 990 μ L DMEM medium was added in the each well and incubated the tissue culture plate in the CO₂ incubator at 37°C. MTT assay was performed to check the cell-viability and proliferation at regular time intervals (days) of 1, 3, 5 and 7 day by measuring the absorbance at wavelength 490 nm using Microplate Reader (TECAN, India).

FESEM analysis of the recellularized matrix was done to confirm the cell adherence and spreading over the scaffold by following the previous protocol [32]. Briefly, on day 7, cell-seeded matrix-constructs were collected and fixed in 4% formalin solution. Further, all the samples were dehydrated using ethanol at 4°C in gradient concentration (50%, 70%, and 100%) for 30 min each and air-dried overnight. Then, the outer surface and cross-sectional images of the recellularized matrices were captured was examined and analyzed by using Image J analysis software.

In vivo wound healing study

For animal wound healing study the protocol was approved by the Institute Animal Ethics Committee (ethical approval number: BT/IAEC/2016/02), Department of Biotechnology (IRB Registration number-563/02/a/CPCSEA), IIT Roorkee, India. In vivo full thickness excisional wound healing experiment were

performed by using procedure as described in the literature [33]. Two months old, 24 healthy albino mice (both sex) having weight 25-30 gm were housed in the Institute animal house and were supplied with food and ad lib water.

Wound construction and graft implantation

Full-thickness excisional wounds were done aseptically under anaesthetic condition on the dorsal back-side of albino mice. Anaesthesia drugs (mg/kg of animal body weight) — diazepam (10 mg) and ketamine (100 mg) doses were administered by the IP route; cleaned the dorsal back side of animals with antiseptic solution (Dettol®); shaved hair and full-thickness excisional wound of diameter 1.5 cm was created by punching method. Subsequently, wounds were cleaned with antiseptic solution and animals were divided into 3 groups (eight animals/group; $n = 8$) according the treatment given to them: Group I (control: Povidone-iodine solution, Betadine®, Win-Medicare Pvt. Ltd., India); Group II (AGDM used as permanent graft); and Group III (H-AGDM used as permanent graft). After treatment, all the wounds were dressed with sterile non-adhesive surgical pad—Combine dressing (KS CARE™, India) and covered with surgical tape (3M Transpore™, North coast medical Inc. USA), for the prevention of dressing omission and proper aeration of wound area. .

Evaluation of wound healing

Macroscopic observation

On post-operative day 3, 7 and 14, colour pictures of the wound site with a digital camera was captured to examine the wound shape, abnormality and colour. Subsequently, the wound contraction and healing area was measured by mapping the size of wound with transparent sheet for tracing the wound area (mm²).

Histology evaluation

On post-operative day 7 and 14, healed tissue biopsies were collected from the wound site to check the neo-tissue formation and proliferation of inflammatory cells. Biopsy samples were fixed in 10% formalin and embedded in paraffin to make blocks. Paraffin coated tissue blocks were sectioned into 5 μ m thick slices and mounted them on glass-slide, followed by H&E staining. Stained samples were observed under phase-contrast microscopy and images were analyzed using Miotic Imaging software (Diagnostic Instruments; Sterling Heights, MI). Inflammatory response was evaluated using the following scale: 0, little or no inflammation; 1, aggregates of inflammatory cells occupying less than 50% of the sample; and 2, aggregates of inflammatory cells occupying 50% or more of the sample [34].

Immunological observations

In vivo immunogenic and allergic responses induced after implantation of matrix —AGDM and H-AGDM, were evaluated by measuring the Immunoglobulins (Igs), Complement component (C3) level and specific inflammatory cell level [25]. Briefly, on post-operative day 7 and 14, blood samples were collected for In-direct ELISA (enzyme-linked immunosorbent assay) analysis and Complete blood counting (CBC) test to measure the level of Igs and immune cells.

Statistical analysis

All quantitative results were measured as mean \pm standard deviation. Experimental data were analyzed statistically by the

analysis of variance (ANOVA) and *p-value (<0.05) for significant difference.

Results and Discussion

FTIR analysis

FTIR spectra of the samples— Honey, AGDM and H-AGDM were analyzed to confirm the possibly chemical interaction and formation of functional of groups present in the matrix (Figure 2). FTIR spectra of honey confirmed the presence of all essential peaks of hydrated carbohydrates—3373 cm⁻¹ for the OH stretching; 2937 cm⁻¹ for CH₂ asymmetrical stretching; 1640 cm⁻¹ for C=O stretching; 1050 cm⁻¹ for C-O stretching. Other peaks at the 2116 cm⁻¹ for C≡C stretching; 1417 cm⁻¹ for OH bending; 920 cm⁻¹ for C-H bending of other components respectively. Similar, many researchers indicates the characteristic peaks of hydrated carbohydrates and others peaks of honey— 3420 cm⁻¹ for OH stretching; 2963 cm⁻¹ and 2910 cm⁻¹ for CH stretching; 1650 cm⁻¹ for C=O; and 1054 cm⁻¹ for CO stretching respectively [16,35,36].

FTIR spectra of AGDM showed peaks— 3473 cm⁻¹ for the NH-stretching and 2923 cm⁻¹ for asymmetrical CH₂ stretching. Other peaks at 1643 cm⁻¹; 1554 cm⁻¹ and 1240 cm⁻¹ confirmed the presence of amide I (C=O); amide II (NH) and amide III (CN) groups respectively, which proves the presence of collagenous structure and functional groups for better cell adherent. The presence all these peaks in the acellular matrix confirm the intact ECM rich matrix after decellularization, similarly as obtained in other studies [26].

In case of the H-AGDM, shifting of peaks were found at 3437 cm⁻¹ for stretching mode of OH, NH groups; 1632 cm⁻¹ for amide I (C=O) and 1052 cm⁻¹ for CO stretching, which confirmed the possible interactions among the functional groups. Other peaks at 2900 cm⁻¹, 2100 cm⁻¹, 1400-1200 cm⁻¹ were disappeared after assimilation of honey with AGDM, which indicated the possible H-bonding or other interaction forms between the ECM molecules and honey components. Other researchers also verified the shifting or disappearance of peaks in different honey composite with other polymers (PEG, PVA,) which results stable cross-linking [16]. Here, it will assumed that possibly the interaction occurs between the free carboxyl and amino group of amino acids and hydroxyl group present in hydrated carbohydrate monomer units (Figure 3).

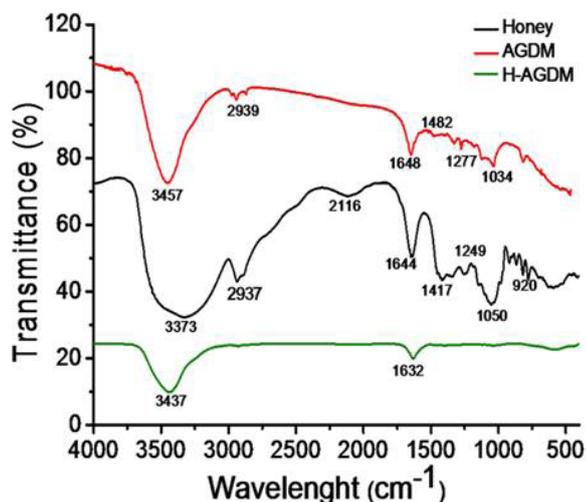


Figure 2: FTIR spectra analysis of honey, AGDM and H-AGDM indicates interaction between them.

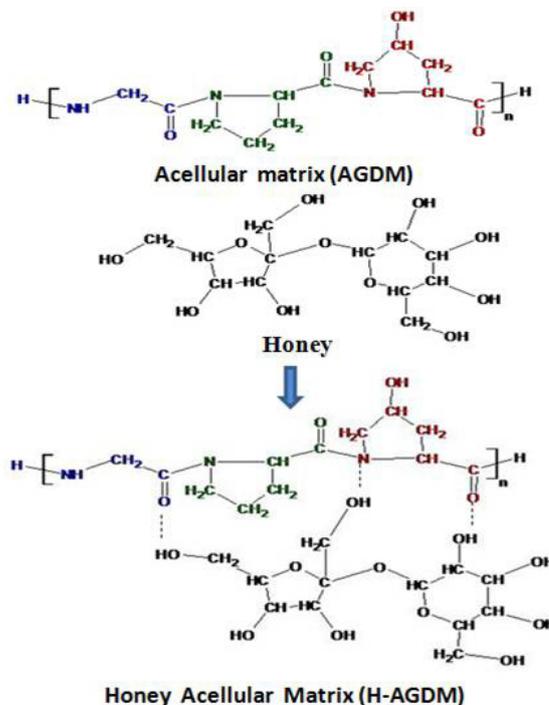


Figure 3: Schematic representation of possible interaction occurs between Honey and AGDM.

Morphological analysis

Ultra-structure of the fabricated AGDM (control) and H-AGDM was determined by FESEM images analysis showed the interconnected 3D porous structure (Fig. 4). The mean porosity and pore size of the acellular were found to be 77.69±26.81µm and 86.56±15.10. However, after modification of acellular matrix with honey showed the subsequently decrease in the pore size and the porosity of the matrix with the increase of concentration of honey— pore size 76 µm to 68 µm and porosity 83 % to 69% . H-AGDM with 5% and 10% concentration showed good porosity and pore-size, which is suitable for cell migration and proliferation. Other researcher proved that the scaffold have microstructure for better cell growth and nutrient diffusion [37].

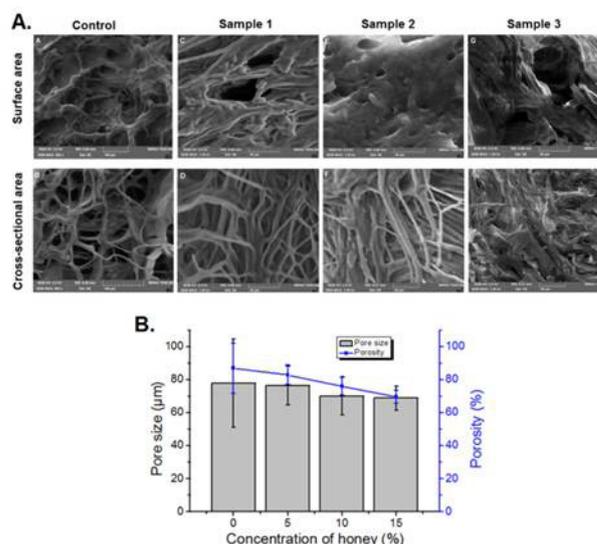


Figure 4: Morphological analysis: (A) FESEM analysis of control (AGDM) and modified scaffold (H-AGDM) at different concentration and (B) Effect of honey concentration on AGDM porosity and pore size.

In vitro biodegradation

The degradability rate of H-AGDM varies under enzymatic and non-enzymatic condition (Figure 5, Table 1). The complete degradation of the control sample (AGDM) and H-AGDM was measured to be similar in non-enzymatic condition (1X PBS) within 125 h. However, in the presence of collagenase enzyme, significant difference was observed between the AGDM (within 75 h) and H-AGDM (within 95 h) degradation time. Honey incorporation with AGDM enhance degradation time, indicates the acellular matrix cross linked with honey molecules. Honey has water sorption capacity (high osmolarity) and solubility, which accelerate the degradation rate and thus swelling due to absence of a compact structure to retain in water [38]. Other researcher observed scaffold modified with honey has slow degradation rate as compare to the native one [39].

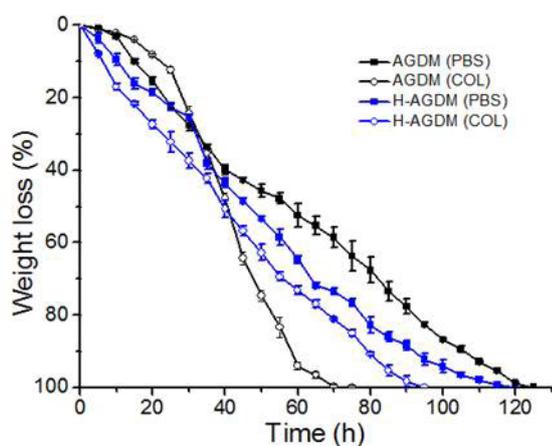


Figure 5: Biodegradation of AGDM and H-AGDM under non-enzymatic (1X PBS) and enzymatic condition (Collagenase- COL) at 37°C

Table 1: Comparison of in vitro degradation of unmodified (AGDM) and Honey modified scaffolds (H-AGDM) under enzymatic and non-enzymatic conditions

Scaffold Type	Honey concentration (%)	Time for complete degradation (h)	
		PBS (1X)	Collagenase (0.05%)
AGDM	0	125	75
H-AGDM	5	130	95
	10	135	110
	15	145	115

Antibacterial activity

Honey have superintendent bactericide activity. The results showed that the H-AGDM significantly inhibits the bacterial growth of both Gram-positive (*E. coli*) and Gram-negative bacteria (*S. aureus*). The antibacterial activity against *E. coli* and *S. aureus* has also been examined for different xenogeneic acellular matrices e.g. porcine liver, urinary bladder, and goat-lung and skin tissue [30,40]. The antimicrobial activity of the ADGM is significantly enhanced after incorporation with honey. Maximum bacterial growth inhibition caused by H-AGDM (10% honey) was up to 60 h for *E. coli*, and 30 h for *S. aureus* (Figure 6). The antibacterial property of AGDM was also reported in earlier

studies [27], had been increased significantly after incorporation of honey. Honey along with the acellular matrix synergistically enhances the antibacterial activity. Naturally, the components present—H₂O₂, enzymes etc., in honey provide bacterial resistance and inhibits the growth of microbes [5,9,11,41]. It was reported that the honey incorporated scaffold effective against gram negative (*S. aureus*) and gram positive (*E.coli*) bacterial strain [12,38,42,43].

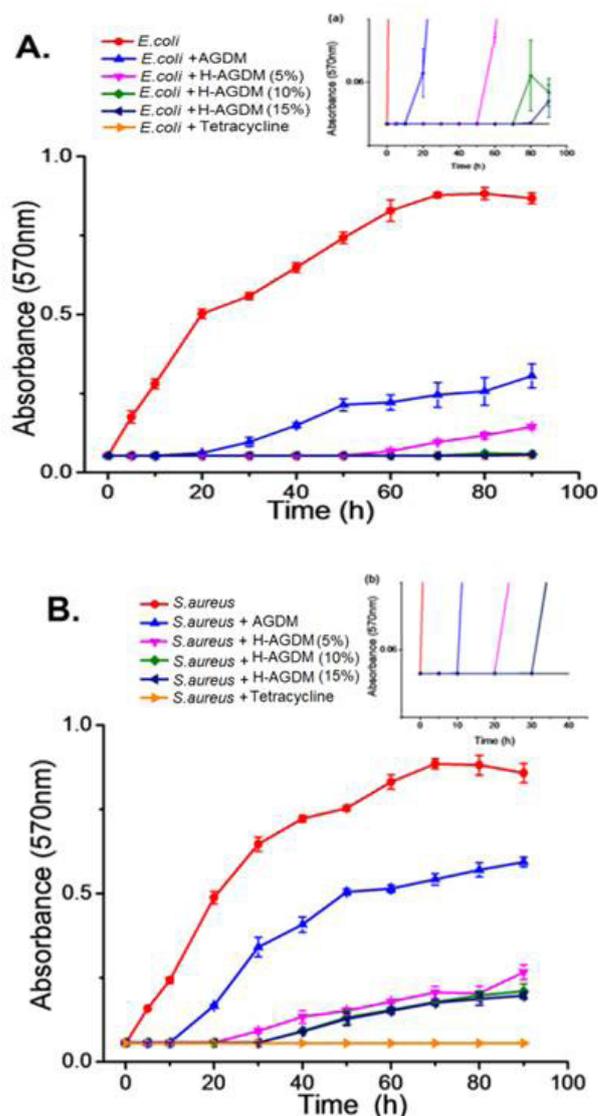


Figure 6: Antibacterial activity of H-AGDM digest: (A) gram negative bacteria *E. coli* and (B) gram positive bacteria *S. aureus* growth inhibition

In vitro biocompatibility

MTT assay confirmed incorporation of honey with acellular matrix (H-AGDM) results better cell growth, proliferation without any cytotoxic-effect. Initially, H-AGDM-cell construct showed low absorbance which indicates the less cell proliferation over the matrix as compared to the control (AGDM) (Figure 7A). However, with the progression time of the absorbance increased and higher than the control sample: cell proliferation over the H-AGDM increased. FESEM images of recellularized construct collected on day 7, showed good cell-adherence and proliferation over the matrix (Figure 7B). Similarly, in other studies polymeric scaffolds modified with honey have positive cell response and good cell attachment with different cell lines [3,16,44,45]. It was reported that the increasing concentration of honey decreases the cell adhesion, at 5% concentration or

below honey significant enhance cell attachment and decreases cytotoxicity [44-46]. Similar, outcomes were reported in the case of honey incorporated acellular matrix (H-AGDM) having 10% honey, which showed constant and controlled cell-growth.

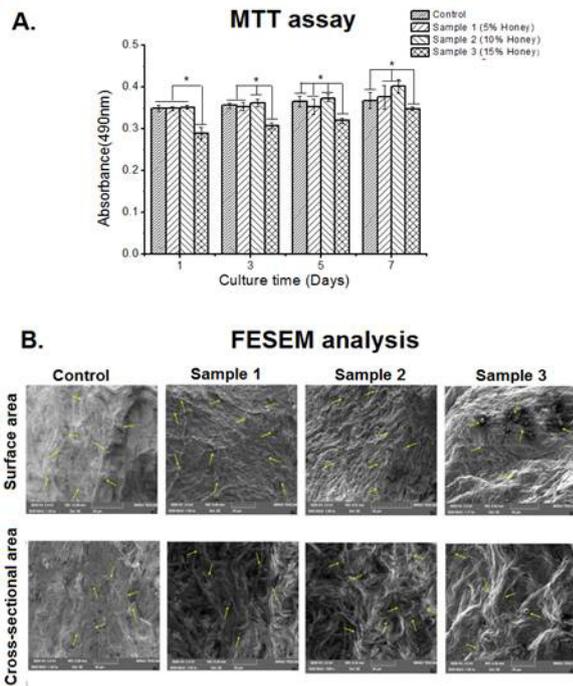


Figure 7: *In vitro* biocompatibility of Honey -AGDM: (A) MTT assay analysis, (*) indicates significant difference between the scaffolds at different concentration of Honey and (B) FESEM analysis of recellularized scaffolds. Yellow arrow indicates neo-ECM and cell over the scaffold

In vivo wound healing study

Honey modified AGDM sample with 10% concentration showed good porosity, biodegradability, antibacterial activity and biocompatibility, which represents its suitability as skin-graft for skin tissue engineering. For *in vivo* biocompatibility and wound healing testing, we used 10% H-AGDM and grafted it on full-thickness wounds created on the dorsal back-side of the albino mice. Wounds treated with H-AGDM showed faster reduction of granulation tissue, re-epithelialization and wound contraction, which indicates accelerated wound healing rate without any inflammatory response (Figure 8A, Table 2). Initially, the wound contraction and healing in H-AGDM group was faster among all the groups, which results closure of wound without any inflammation (Figure 8B). On post-operative day 7, H-AGDM treated wounds, showed significantly epithelization with intact ECM deposition. With the progression of time, on post-operative day 14, the deposition of collagen rich ECM with thick intact outer epidermal layer covering was form in H-AGDM group as compare to the other group (Figure 8C). Several studies of wounds treated with honey or honey based grafts showed early epithelization, increased deposition of the collagenous matrix, accelerated wound contraction and enhanced autolytic debridement, which results in improved wound healing rate with reduced scarring at the wound site [13,15,45,47-49].

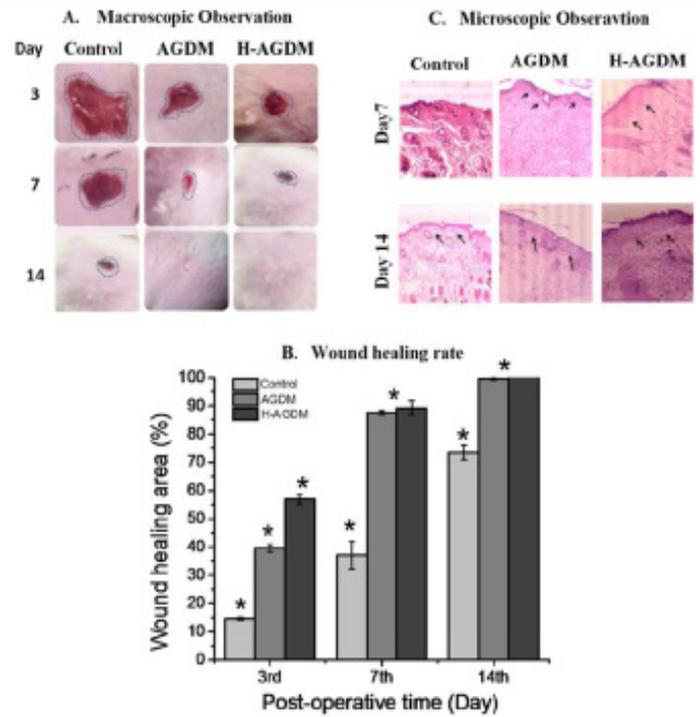


Figure 8: Wound healing study of H-AGDM: (A) Macroscopic observation of wounds at regular time interval. Wound area indicated by blue dotted line; (B) Wound healing rate (%) at regular time interval, (*) indicates significant difference between the groups, and (C) Histomorphological analysis of healed wound tissue by microscopic observation of H&E stain tissue image. Arrow indicated neo-tissue formation.

Table 2: Inflammatory response at the wound site of different groups at day 7 and 14 after post-operative treatment.

Day	Control	AGDM	H-AGDM
7	1.8	0.50	0.35
14	1.25	0	0

Here, the inflammatory response scored as: value “0” for little or no inflammation, “1” for moderated or less inflammation and “2” for extensive.

During wound healing prolonged inflammation, results delayed healing and formation of chronic non-healing wound [50]. On post-operative day 7 the WBC counts of H-AGDM was similar as AGDM, but on day 14 the WBC counts were significantly reduce in H-AGDM group (Figure 9A). However, the lymphocytes counts in H-AGDM group were significantly low compared to other groups and reduced with the progression of time from day 7 to 14 (Figure 9B). The monocytes counts of AGDM and H-AGDM group treated animals were similar but significantly lower than other groups (Figure 9C). The humoral mediated immune response measured by indirect ELISA test indicates less proliferation or insignificant proliferation of IgG, IgM and C3 formation in H-AGDM treated group (Table 3). H-AGDM treated wounds showed controlled immune response generation which results better healing. Chronic and burn wounds treated with honey cross linked hydrogel showed significant decrease in inflammatory response [20,51]. Honey enhanced the immune response, stimulates monocytes proliferation, which releases cytokines for better wound healing [7,41,52]. Leong and co-workers demonstrated that wounds treated with honey have reduced leukocytes infiltration [53]. Honey has natural superior anti-inflammatory property, provides catabolic environment, have high sugar content along with free amino acids, which

results faster re-epithelization and dense connective dermal tissue layer formation at wound site [4,54-56]. Farzadinia and co-worker, used honey-milk-aloe vera ointment for wound healing, on post-operative day 28 wounds were healed with less inflammation and reduce scarring [57]. Blood and serum test of treated animal were collected on post-operative day 7 and 14, showed H-AGDM group animal have significantly less proliferation of inflammatory cell—WBC, Lymphocytes as compare to the other groups.

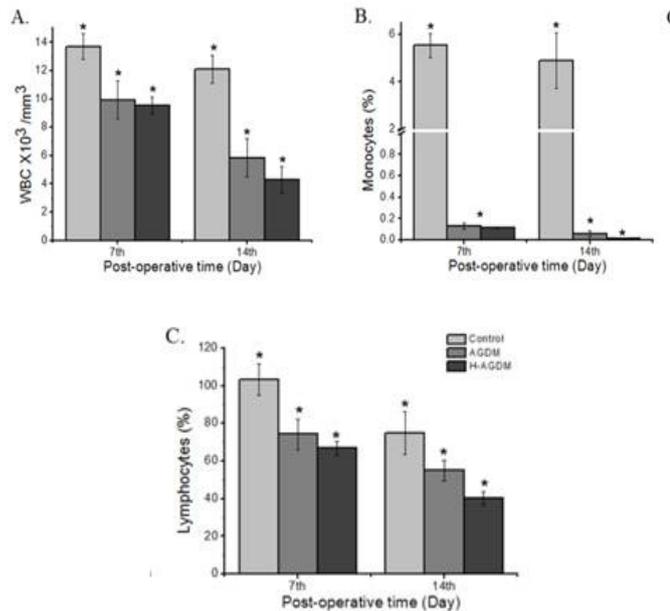


Figure 8: Inflammatory response of Honey-AGDM by CBC analysis (A) WBC count, (B) Monocyte count and (C) Lymphocyte count. (*) indicates significant difference among the groups.

Table 3: Immunogenic response induced in skin wound in mice model for different Groups i.e. control (Group I), AGDM (Group II) and H-AGDM (Group III) at day 7 and 14.

Day	7	14	7	14	7	14
Type	IgG		IgM		C3	
Control	+	-	+	-	+	-
AGDM	-	-	-	-	-	-
H-AGDM	-	-	-	-	-	-

Here + for high level and – for low level of Ig and C3 molecule

Conclusion

Honey incorporated acellular dermal matrix was successfully evaluated as natural graft for accelerating the wound healing process. The H-AGDM having 10% honey concentration is the best composition with good ultrastructure, antibacterial resistivity, biodegradability, biocompatibility and anti-inflammatory response which results faster wound healing. Based on this study, we concluded that the honey modified acellular graft is potential applicable for wound healing. Therefore, H-AGDM could be used as a permanent antibacterial graft for wounds healing and tissue regeneration.

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