Formulation and Evaluation of 7, 8- Dihydroxy Flavone Loaded Topical Hydrogel Scaffold

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ABSTRACT
Burnt skin tissue defects pose a significant challenge for physicians to manage owing to the limited skin regeneration potential. Various conventional approaches (i.e. autografts and allografts) have been endorsed for the restoration of tissue defects with inadequate results. However, skin tissue engineering techniques were appeared as an optimistic approach to improve the skin tissue regeneration. Hydrogels offering advantages over the current treatment modalities of the damaged skin tissue were proposed as an advanced and promising therapeutic approach for effective skin tissue regeneration. In general, an ideal hydrogel scaffold used for burnt skin tissue regeneration must show excellent biodegradability, biocompatibility and bioadhesive characteristics. Moreover, among the variety of several hydrogel scaffolds for skin tissue regeneration, topical hydrogels developed from both natural and synthetic polymers are likely to fulfil these above characteristics. In this study Novel 7, 8- Dihydroxy flavone loaded PVA/Agar hydrogel was developed using the facile physical cross-linking technique. Further, the characterization studies confirmed that this hydrogel scaffold possess an ideal porous cross linked structure along with excellent Hemocompatibility, cellular proliferation and controlled drug release. Histopathological staining’s provided further evidence for the reepithelization, and revascularization potential of the developed hydrogel scaffold. Moreover, our research findings revealed that the 7, 8-Dihydroxy flavone loaded PVA/Agar hydrogel is hemocompatible and capable of burnt skin tissue repair and also possesses significant potential for skin tissue engineering.

INTRODUCTION
Hydrogels offering advantages over the current treatment modalities of the damaged skin tissue were proposed as an advanced and promising therapeutic approach for effective skin tissue regeneration. In addition, hydrogels plays a prominent role in mimicking the characteristics of the skin extracellular matrix and vital for fastening the skin tissue regeneration process. Even though several therapeutic procedures including autograft and allograft, were available to restore the damaged skin extracellular matrix, these procedures often do not result in development of durable and biomimetic skin tissue (Du et al., 2015; Dimatteo et al., 2018).
In general, an ideal hydrogel scaffold used for burnt skin tissue regeneration must show excellent biodegradability, biocompatibility and bioadhesive characteristics. Moreover, among the variety of several hydrogel scaffolds for skin tissue regeneration, topical hydrogels developed from both natural and synthetic polymers are likely to fulfil these above characteristics. More importantly, the natural and synthetic polymers such as PVA, PVP, Dextran, Agar, Chitosan, and Gelatin because of the excellent biomimetic property made them an ideal candidate for the design and development of hydrogel scaffold. Further hydrogel were considered as a promising source to repair damaged skin tissue effectively due to their excellent porosity and moisture retaining capabilities. However, the most widely used technique in skin tissue regeneration requires an acceptable combination of drug molecule, a biocompatible, and biodegradable polymeric hydrogels that facilitate the formation of skin extracellular matrix. Moreover, the favourable outcome of skin tissue regeneration depends on combined characteristics, of the drug loaded polymeric hydrogel scaffolds. In this work, we fabricated the 7, 8-Dihydroxyflavone loaded PVA/Agar hydrogel for effective skin tissue regeneration (Branski et al., 2008; Homann et al., 2007; Alapure et al., 2018).

7, 8- Dihydroxy flavones (7, 8-DHF) obtained from Godmania aesculifolia, Tridax procumbens, and primula tree leaves acts as a tropomyosin-receptor-kinase B (TrkB) agonist. Recently 7, 8-DHF demonstrated a distinct activity other than neurogenesis. It could also promote anti-inflammatory and antioxidant activity. Hence based on these findings we hypothesised that combination of PVA/Agar hydrogel and 7, 8- Dihydroxy flavone shows the synergistic effect on the burn wound healing process (Park et al., 2012; Han et al., 2014).

However, PVA/Agar based hydrogel for burnt skin tissue regeneration in this work, since agar is a natural polymer obtained from certain species of algae (such as Agarophytes and Rhodophytes); shown excellent biodegradability and biocompatibility hence it played a prominent role in wound healing applications (Saraswathy et al., 2012). In our previous studies agar was used in the development of the hydrogel for skin tissue regeneration. Further, our research group also reported that PVA/Agar hydrogel scaffold stimulated the reepithelization with excellent angiogenesis in case burnt wound model (Uppuluri and Shanmugarajan, 2019).

Due to its ideal non-cytotoxic, biomimetic, biocompatible, and mechanical characteristics polyvinyl alcohol (PVA) played a vital role in various biomedical applications. More importantly in the current study PVA significantly increased 7, 8-Dihydroxyflavone retention on the damaged skin tissue owing to its excellent bioadhesive property. Furthermore, the PVA/Agar hydrogel also provided the environment similar to that of host tissue and this in turn favours the enhanced angiogenesis in case of burnt skin tissue model (Tao et al., 2019; Kong et al., 2019).

In our previous study, we demonstrated the amorphous, chemical interactions and swelling characteristics of the PVA/Agar hydrogel. The current study relate to the development of the hydrogel scaffolds contributing the controlled release of 7, 8-Dihydroxy flavone, which can be applied effectively at the burnt wound site due to its ideal mechanical properties. Besides our proposed hydrogel demonstrated the ideal Hemocompatibility, swelling characteristics, and wound healing potential. Furthermore, in the present investigation 7, 8- Dihydroxy flavone loaded hydrogel was introduced to the burnt skin tissue defects and evaluated for its efficacy by performing various in-vitro and histopathological studies for accelerated skin tissue regeneration.

MATERIALS AND METHODS

Materials
7, 8- Dihydroxy flavone was purchased from Tokyo Chemical India Pvt. Ltd, Chennai, India. Agar-agar was purchased from the HI media. Industrial grade PVA was purchased from Spectrum Chemicals Pvt Ltd, Kochi, India. All the samples were obtained from genuine source and were used as such.

Preparation of 7, 8- Dihydroxy flavone loaded hydrogel
PVA/Agar hydrogels were prepared as previously reported (Uppuluri and Shanmugarajan, 2019). Briefly, 1% w/w of PVA, 3% w/w Agar was added to the distilled water and stirred under the reflex (using magnetic stirrer) at 80°C for two hours. Later 20mg of the drug was added to above solution and stirred at 80°C for 90 minutes. Finally, the sample was placed in room temperature for 24 hours in order to get it stabilised.

Swelling Studies
PBS (pH 7.4) was used in this analysis to analyze the swelling behaviour of formed hydrogel scaffolds. Both PVA/Agar hydrogel and 7, 8-Dihydroxy flavone loaded hydrogel were initially freeze-dried, later precise amounts (i.e. 2 ml) of the formed hydrogel scaffolds and immersed for 24 hours in 10 ml of PBS. Finally, both samples were isolated from the PBS after 24 hours and weighed easily. Finally the %
swelling was calculated by using the following equation:

\[
% \text{Swelling} = \left( \frac{m_1 - m_0}{m_0} \right) \times 100
\]

Where, \( m_0 \) is the weight of freeze-dried hydrogels and \( m_1 \) is the weight of freeze-dried hydrogels after 24 hours.

**FTIR**
7, 8-Dihydroxy flavone hydrogel FTIR spectra were analyzed for the presence of chemical constituents using an ATR (attenuated total reflectance)-equipped Perkin Elmer Spectrum 100 spectrometer with a resolution of 4 cm\(^{-1}\). The spectrum was reported using the KBr pellet method in the range of 400-4000 cm\(^{-1}\).

**FESEM**
For the study of PVA/Agar hydrogel and 7, 8-Dihydroxy flavone loaded hydrogel morphologies, a field emission scanning electron microscope (S-4700, Hitachi Limited, Japan) was used. On an aluminium mount, the freeze-dried hydrogel samples were mounted, sputtered with platinum and then scanned at an accelerating voltage of 20 kV.

**MTT Assay**
The cell viability of fibroblasts within hydrogels was investigated based on the reduction of the MTT reagent and the formation of formazan due to presence of enzyme dehydrogenases in the mitochondria. Further at the density of 1 \( \times 10^4 \) 3T3 murine fibroblast cell line were seeded on the developed hydrogel scaffolds in DMEM/F12 containing the FBS (10 % v/v), penicillin (100 unit/ml), streptomycin (100 \( \mu \)g/ml) and incubated in the 5% CO\(_2\) at 37°C. During 1\(^{st}\) and 3\(^{rd}\) day 150 \( \mu \)l of MTT was added to each well after removal of culture medium from 96 well plate. Later, MTT solution was withdrawn after 4 h, and replaced with 1ml DMSO solution for dissolving formazan crystals. Finally, the formazan solution’s optical density was measured on a microplate reader Anthos 2020 (Biochrom, Berlin, Germany) at 570 nm. This assay was repeated three times.

**In-vitro release studies**
UV Visible Spectroscopy was utilized to determine the release of 7, 8-Dihydroxy flavone from the PVA/Agar hydrogel. 7, 8-Dihydroxy flavone loaded PVA/Agar hydrogel (1 mL) were loaded to simulated body fluid (SBF) (5 mL) and the samples were shaken at 40 rpm in an incubator shaker at 37°C. The supernatants were extracted and centrifuged at different time intervals, and the absorption intensity of supernatants was measured at 350 nm, to determine the amount of released 7, 8-Dihydroxy flavone.

**Hemocompatibility**
In this study, 2.5 ml of prepared hydrogels (i.e. PVA/Agar hydrogel and 7, 8-Dihydroxy flavone loaded hydrogel) were added to 2 % off-fiber rat erythrocyte suspension and incubated at 37°C. After incubating for 3 h, the erythrocyte suspension was centrifuged for 10 min at 2000 rpm. Then the erythrocyte suspension supernatant was collected and observed at 540 nm on a UV /Vis spectrophotometer (Lambda 35, Perkin Elmer), for determining the % hemolysis. Finally, the % Hemolysis was represented by the following equation:

\[
% \text{Hemolysis} = \left( \frac{TS - NC}{PC - NC} \right) \times 100
\]

Here TS represents Test sample (i.e. PVA/Agar hydrogel; 7, 8- Dihydroxy flavone loaded hydrogel); NC represents Negative Control; PC represents Positive Control. Whereas, in this study normal saline and distilled water was used as negative and positive control.

**Histopathological study**
With 10 % buffered formalin, the extracted wound tissues were fixed and deposited in paraffin blocks. The samples were cut with a microtome (Leica, Germany) to 5 \( \mu \)m tissue sections. Then tissue sections were dewaxed with a mixture of xylene and ethanol, and were stained with H&E stain accompanied by examination under a light microscope.

**Statistical analysis**
Origin pro software (Version 9) was used to analyse the results obtained. In addition, all the results obtained were evaluated using the row statistics and a two-sample t-test on rows, the data was expressed as a mean ± standard deviation. The results showing \( P < 0.05 \), \( P < 0.01 \), and \( P < 0.001 \) were also considered statistically significant.
RESULTS AND DISCUSSION

Swelling studies
The swelling behaviour of the PVA/Agar hydrogel and 7, 8- Dihydroxy flavone loaded hydrogel were analysed from their water uptake value. Moreover, there exists a relationship between the swelling and porosity i.e. higher the porosity higher will be the swelling rate. However, the pore size of these developed hydrogels plays a significant role in sustained drug release, cellular attachment and proliferation (Ahmadi et al., 2015). Further after 24 hours of swelling in case of the results it was identified that PVA/Agar hydrogel shows swelling percentage of 900 ± 12% and whereas, the 7, 8- Dihydroxy flavone loaded hydrogel shows swelling percentage of 800%3 ± 12%.

FTIR
The substantial evidence for the existence of 7, 8- Dihydroxyflavone in the hydrogel was provided by the FTIR spectroscopy (Chen et al., 2020). Whereas,
the results of our previous findings revealed that in the case of FTIR spectra of PVA/Agar hydrogel there exists a significant interaction among the PVA and agar molecules. Further, the FTIR spectrum of the 7, 8-Dihydroxy flavone loaded hydrogel (Figure 1) exhibited distinguishing peaks at 3370, 1643, 1012 cm⁻¹, coincided with stretch bands of OH, C=O, C-O groups. It was observed that there was slight shift in the characterisation peaks of the 7, 8-Dihydroxy flavone loaded hydrogel when compared to PVA/Agar hydrogel peak and this in turn proved that there is significant interactions between the 7, 8-Dihydroxy flavone and excipients present in the developed hydrogel.

Figure 4: MTT assay histogram of developed hydrogel scaffold after 24 and 72 hours post cell seeding. Where, mean ± SD, n= 3, * p < 0.05, **p < 0.01, and ***p < 0.001.

Figure 5: Cumulative release profile of 7, 8-Dihydroxy flavone from the PVA/Agar hydrogel in SBF at 37°C.

FESEM
The 3D cross linked structure of the developed hydrogels were characterized and product analysis was conducted using the FESEM (Wakhet and Singh, 2015). Further the incorporation of the 7, 8-Dihydroxy flavone into interconnected porous networks of the developed hydrogels shown ideal spreading of the 7, 8-Dihydroxy flavone within developed scaffolds. Furthermore, the 3D networking structure of the drug loaded hydrogel confirmed that addition of 7, 8-Dihydroxy flavone did not alter the 3D interconnected network structure of the PVA/Agar hydrogel scaffold. Figure 2 represents the FESEM image of the PVA/Agar hydrogel scaffold and Figure 3 represents the FESEM image of the 7, 8-Dihydroxy flavone loaded hydrogel scaffold.

MTT Assay
To determine the cell viability and proliferation the fibroblast cells were seeded on the developed hydrogels. Figure 4 on day 1 showed no significant difference in fibroblast proliferation of both PVA/Agar hydrogel group and 7, 8-Dihydroxy flavone loaded hydrogel group. However on day 3 the fibroblast proliferation was significantly increased (P<0.001) in 7, 8-Dihydroxy flavone loaded hydrogel compared to the PVA/Agar hydrogel. MTT assay demonstrated that 7, 8-Dihydroxy flavone loaded hydrogel promotes the cellular proliferation compared to other groups, and this in turn demonstrated the positive effect of 7, 8-Dihydroxy flavone. In fact, literature evidences suggest that both phenolics and flavonoids promote the fibroblast proliferation which in line with our studies (Zain et al., 2020). Furthermore the findings also indicated that the prepared hydrogel did not show any cytotoxicity effects and improved proliferation potential on the cells.

Invitro release study
Present invitro study findings were related to controlled release mechanism, in which the drug molecule incorporated in hydrogel matrix initially swells after coming in contact with PBS and later released the drug slowly from the interconnected gel network (Kim et al., 1992). As shown in Figure 5 the amount of the released 7, 8-Dihydroxy flavone...
reached $10.86\% \pm 1.32$ and $23.45\% \pm 2.73$ at 4h and 8h after initiation of the study. Further, it is followed by the controlled release of $83.67\% \pm 4.36$ for over a period of 15 days, yet providing the sufficient time for the release of drug on the burnt skin tissue portions.

**Hemocompatibility studies**

In the light of fact, there exists a relationship between the hemolysis rate and hemocompatibility of the developed hydrogel scaffolds (i.e. higher the hemocompatibility lesser will be the rate of hemolysis) of the developed hydrogel scaffolds. The affinity of the developed hydrogel scaffolds towards the blood cells or erythrocytes was essential for the skin wound healing. Moreover, the hydrogel interaction is an early stage event that demonstrates the body reactions like inflammation, after its implantation into the body (Hoque et al., 2017). As illustrated in Figure 6 Hemolysis caused by the developed PVA/agar Hydrogel and 7, 8-Dihydroxy flavone loaded hydrogel were found to be less than 5%, and that in turn demonstrates the excellent Hemocompatibility of these developed hydrogel scaffolds.

**Histopathological study**

Paraffin embedded $5\mu m$ tissue section samples of negative control, PVA/Agar hydrogel and 7, 8-Dihydroxy flavone loaded hydrogel treated groups was prepared for H&E staining. As shown in Figure 7 at day 5 the burnt skin tissue defects treated with 7, 8- Dihydroxy flavone loaded hydrogel shown the moderate rate of inflammatory cells infiltration and minimal amount of tissue necrosis. Whereas, in case of negative control highest rate of inflammatory cells infiltration was recorded when compared to PVA/Agar hydrogel treated group. Further during day 10 neovascularization and reepithelization was observed in case of tissue sections treated with the 7, 8- Dihydroxy flavone loaded hydrogel. Whereas, the PVA/ Agar hydrogel treated group during 10th day shown moderate rate of inflammatory cells infiltration and granular tissue formation when compared to the negative control. Finally 15th-day tissue sections clearly revealed that 7, 8- Dihydroxy flavone loaded hydrogel shown excellent reepithelization and revascularization when compared to the PVA/Agar hydrogel and negative control. The results of histopathological staining revealed that burnt skin tissue regeneration was better in the 7, 8- Dihydroxy flavone loaded hydrogel treated group than in the PVA/Agar hydrogel and Negative control treated groups.

**CONCLUSIONS**

In this study, 7, 8- Dihydroxy flavone loaded PVA/Agar hydrogel was developed with facile pro-
cedures. The topical hydrogel scaffolds were analysed as having an ideal 3D cross linked structure, swelling. In vitro Hemocompatibility and 7, 8-Dihydroxy flavone release were further evaluated. Histopathological staining revealed ideal outcome of the 7, 8-Dihydroxy flavone loaded hydrogel with native extracellular matrix like skin tissue regeneration at 15 days. Hence we propose that the 7, 8-Dihydroxy flavone loaded hydrogel shows significance for clinical applications and should further be analysed for its future importance in the skin tissue engineering.

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Conflict of interest

The authors declare that they have no conflict of interest for this study.

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