Original article

The wound healing potential of 2,3 dimethylquinoxaline hydrogel in rat excisional wound model.

Abstract

Background: 2,3-dimethylquinoxaline (DMQ) is a naturally occurring compound with documented antifungal activity. It has good physicochemical and pharmacokinetic characteristics and showed a good safety profile in both in vitro and animal toxicity studies.

Methods. In silico prediction of pharmacokinetic parameters of DMQ and its potential targets using siss-ADME software. Formulation of DMQ 1% hydrogel using hydroxypropyl methylcellulose. Conducting a pilot study in a limited number of healthy Wister rats using the excision wound model. On day 7, the mean percentage closure of the wound area was determined, the animals were sacrificed, and the skin was isolated for histology and assessment of a panel of inflammatory markers TNF-α, IL-6 IL-β1, hydroxyproline and tissue growth factor by ELISA.

Results and discussion. In silico study revealed that DMQ is likely to target Chemokine (C-C motif) receptor suggesting potential wound healing activity. Almost complete wound healing (97 %) was observed after treatment with DMQ 1 % hydrogel for 7 days. The histological study confirmed marked attenuation of wound-induced histological changes. There was a marked reduction in TNF-α, IL-6 IL-β1 and NF-κB. levels. These data suggest the anti-inflammatory effect of DMQ.

Conclusion DMQ has potential skin wound healing ability likely due to its anti-inflammatory mechanism. Further study is needed to confirm these preliminary findings and explore other molecular mechanisms.

Keywords: 2,3-dimethylquinoxaline, hydrogel, wound healing, inflammatory biomarkers**,** siss-ADME,

Introduction

Wounds are defined as harm to the skin's structural integrity brought on by many causes such as burns, cuts, scalds, and lesions such as foot complications of diabetes (Young and McNaught 2011). Improper management of wounds is associated with many complications such as infections, wound dehiscence, inflammation, scarring, and improper angiogenesis and regeneration (Chadwick, Heath et al. 2012, Aldridge 2015).

It is anticipated that chronic wounds will continue to be a significant challenge because of the ageing population, and the ongoing increase in diabetes, obesity, and infections around the globe (Sen 2021). These complications increase morbidity and mortality, negatively impact

patients' quality of life, and place a tremendous financial load on healthcare systems. Different approaches are used to promote wound healing, including cell therapy, gene therapy, growth factor delivery, wound dressings, and skin grafts. However, none of these modalities is effective for all kinds of wounds (Kolimi, Narala et al. 2022). Moreover, most sophisticated approaches are expensive and not readily available in developing countries. As a result, it is essential to create newer and creative therapy methods for multifaceted therapeutic regimens for chronic wounds.

The use of herbal natural products as possible helpful agents in the process of wound healing has been the subject of an increasing number of research papers (Ramalingam, Chandrasekar et al. 2022). Indeed, the main benefits of using these herbal remedies are their low cost, availability, and minimal side effects. In this context, the efficacy of phytochemicals in the management of wounds is attributed to their antimicrobial, anti-inflammatory, and antioxidant effects. Some phytochemicals also influence the mitogen-activated protein kinase (MAPK) and transforming growth factor-beta (TGF-) signalling pathways (Thangapazham, Sharad et al. 2016, Shah and Amini-Nik 2017, Binsuwaidan, Elekhnawy et al. 2022, Wan, Chen et al. 2022, Criollo-Mendoza, Contreras-Angulo et al. 2023). Examples of extensively studied phytochemicals for potential wound healing include flavonoids (Zulkefli, Che Zahari et al. 2023). The wound-healing activity of other active ingredients such as alkaloids, saponins, and terpenoids was also reported (Balkrishna, Sakshi et al. 2022).

Some quinoxaline derivatives reported to possess wound-healing potential (Geefhavani, Reddy et al. 2012). Quinoxaline derivatives were patented for anticancer and antimicrobial activity (González and Cerecetto 2012). Charles and Minakiri reported the natural presence of 2,3-dimethylquinoxaline (DMQ) (Fig 1) in the *Chromolaena odorata* plant (Charles and Minakiri 2018). They suggested that the observed antimicrobial activity of *Chromolaena odorata* could be attributed to the presence of DMQ. Alfadil and his colleagues confirmed that DMQ had a broad-spectrum antifungal activity (Alfadil, Alsamhan et al. 2021). It is also effective against Madurella Mycetomatis (Alfadil 2021). Using the fuzzy adaptive least-squares (FALS) method, Moriguchi and his coworkers predicted that DMQ is non-carcinogenic (Moriguchi, Hirano et al. 1996).

The lipophilicity of DMQ was determined by the distribution coefficient ($logD_{7.4}$) between n-Octanol and phosphate buffer at pH 7.4. It was significantly (P <0.05) higher than

tolbutamide and lower than ketoconazole. DMQ had a significant (P <0.05) high permeability compared to antipyrine, atenolol, talinolol, and estrone 3-sulfate. It showed a good safety profile in both in vitro and animal toxicity studies. An acute dermal toxicity study in rats revealed the safety of topical hydrogel of DMQ (Alsamhan 2022).

Therefore, the present study aims to conduct in silico prediction of DMQ potential pathways influencing wound healing and to conduct a pilot to explore its efficacy for accelerating skin wound healing in an animal model.

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2. Materials and Methods

Materials

2,3-Dimethyquoxaline (DMQ), (Quinoxaline-2,3-dimethyl), (Cat. No. D184977; Chemical Abstracts Service, CAS #2379-55-7), Sigma-Aldrich (D184977 - Taufkirchen, Germany). Hydroxypropyl methylcellulose (HPMC) and Methanol were pharmaceutical grades.

Methods

In Silico Prediction of potential pathways influencing wound healing :

Firstly, we predicted potential targets of DMQ and conducted a pathway enrichment analysis using Swiss Target Prediction (http://www.swisstargetprediction.ch/, accessed on 2 April 2023), employing the reported method (Sun, Wang et al. 2021). The SD file type of DMQ was downloaded from PubChem. SwissADME free web tools (http://www.swissadme.ch) were used to compute ADME and different related parameters for drug-likeness calculations as well as topological polar surface area (TPSA) (Daina, Michielin et al. 2017).

Preparation of plain hydrogel:

The plain HMPC hydrogel (2% w/w) 10 was made by transferring 2 g of HPMC to an appropriate beaker, adding 98 g of purified sterile water, covering the container, and leaving it for 24 hours to create the gel.

Formulation and evaluation of the DMQ hydrogel (1%w/w):

0.5 g of DMQ was dissolved in 4.5 g of methanol, the solution was added gradually to 45 g of the plain HPMC gel, with continued gentle mixing. Both plain and medicated gels were

stored in suitable glass jars and stored at room temperature. The colour, homogeneity, consistency, and spreadability of the formulation were all visually examined. The clarity was evaluated using natural light, and all macroscopic analyses were performed in comparison to the plain hydrogel.

Animal study:

The Biomedical Ethics Committee approved the experimental protocol at King Abdulaziz University (KAU) and the National Committee of Bioethics approved the study protocol vide Approval No (PH-1443-76). Animal handling was performed in strict compliance with the ethical guidelines for treating animals as defined by KAU and NCBE.

Wound Excision

Male Wistar rats weighing about 200±20 g were used. Animals were kept at a relative humidity range of 40–70% and a temperature of 22 ± 2 ^{o}C, under a 12/12 h light-dark cycle. Ketamine (90 mg/kg) was used to sedate the rats. 75% ethanol was utilized to sterilize their shaved skin on the dorsal region. Following that, a 100 mm^2 area of skin on the rat's dorsal surface was excised. After wound disinfection, the pain was minimized by injecting 4 mg/kg of lidocaine hydrochloride (2%) with 1:80,000 epinephrine (SC). Each rat was placed in its cage and given standard chow pellets and unrestricted water. The rats were randomly divided into three groups: Group 1: healthy control (no wound) (n=3), Group 2: plain hydrogel (n=3), and Group 3: DMQ hydrogel (n= 3). Topical treatment was done as soon as the wound was created and continued once daily for seven days. Wound diameters (WD) and lesions were photographed on days 0 and 7. On day 7, one rat from each group (selected randomly) was decapitated and the injured skin was dissected. For histology studies, a part of the skin was preserved in 10% neutral formalin. The other piece was cleansed with ice-cold saline before being instantly frozen in liquid nitrogen and kept at -80 ^{o}C for biochemical, immunological, and other biomarker analyses in skin tissue homogenate.

Wound measurement:

The percentage of wound contraction was determined according to the following formula:

Wound contraction % = (WD on day 0 − WD on day 7)/(WD on day 0) × 100.

Preparation of tissue homogenate :

The frozen skin specimens were delicately blotted between filter papers and weighed. Then 10% (w/v) homogenates were prepared in phosphate-buffered saline (PBS, 50 mM potassium phosphate, pH 7.4, ice-cooled) before spinning at 3000 rpm for 20 minutes at 4 ^{o}C. the supernatant was aspirated and used for biochemical assessment of biomarkers.

Analyses of oxidative stress markers:

The following biochemical parameters (oxidative stress) were assessed in skin tissue homogenate Assessments of the total protein, catalase (CAT), superoxide dismutase (SOD), Glutathione (GSH), and malondialdehyde (MDA) were performed using biochemical ELISA kits (Cayman® Chemical, Ann Arbor, MI, USA). based on the instructions provided by the manufacturer.

Analyses of pro-inflammatory and other biomarkers:

The following pre-inflammatory markers were assessed in skin tissue homogenate using ELISA Kits: Tumor necrosis factor alpha (TNF-α), Interleukin 6(IL-6), Interleukin B1(IL-β1) and Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB). Transforming Growth Factor Beta 1 (TGF ß1), and hydroxyproline based on the instructions provided by the manufacturer (Cayman® Chemical, Ann Arbor, MI, USA).

Histological examination

Skin tissues were preserved in a 10% neutral formalin solution for 24 hours before being dehydrated and embedded in paraffin. Tissue sections were cut at four µm-thickness, stained with hematoxylin and eosin (H and E), and examined using a light microscope (Nikon, Eclipse 80i, Japan).

Results

DMQ targets chemokine and PLC

We found that the Chemokine (C-C motif) receptor 1 was listed as one of the top 20 signalling pathways. Chemokine (C-C motif) receptor 1 and phosphatidylinositol phospholipase C activity were key predicted factors responsible for cellular responses to transforming growth factor beta stimulus, tumor necrosis factor, and cell chemotaxis(Rees, Greaves et al. 2015, Zhu, Jones et al. 2018). The structure of DMQ, its Silico Predicted Pharmacokinetic (PK) parameters , namely : lipophilicity(LIPO), size, polarity (POLR), solubility (INSOLU), flexibility (FLEX) , saturation. (INSATU). Topological Polar Surface Area (TPSA) are presented in (Fig 1) and (table 1). These data suggest that the drug likely to show good oral absorption and bioavailability. The Log Kp cm/s of DMQ (skin permeability) is better than Betamethasone and comparable to Ketoconazole . DMQ showed relatively low TPSA of about 26 A^2 suggesting good passage through biological membrane including blood brain barrier (Shityakov, Neuhaus et al. 2012)

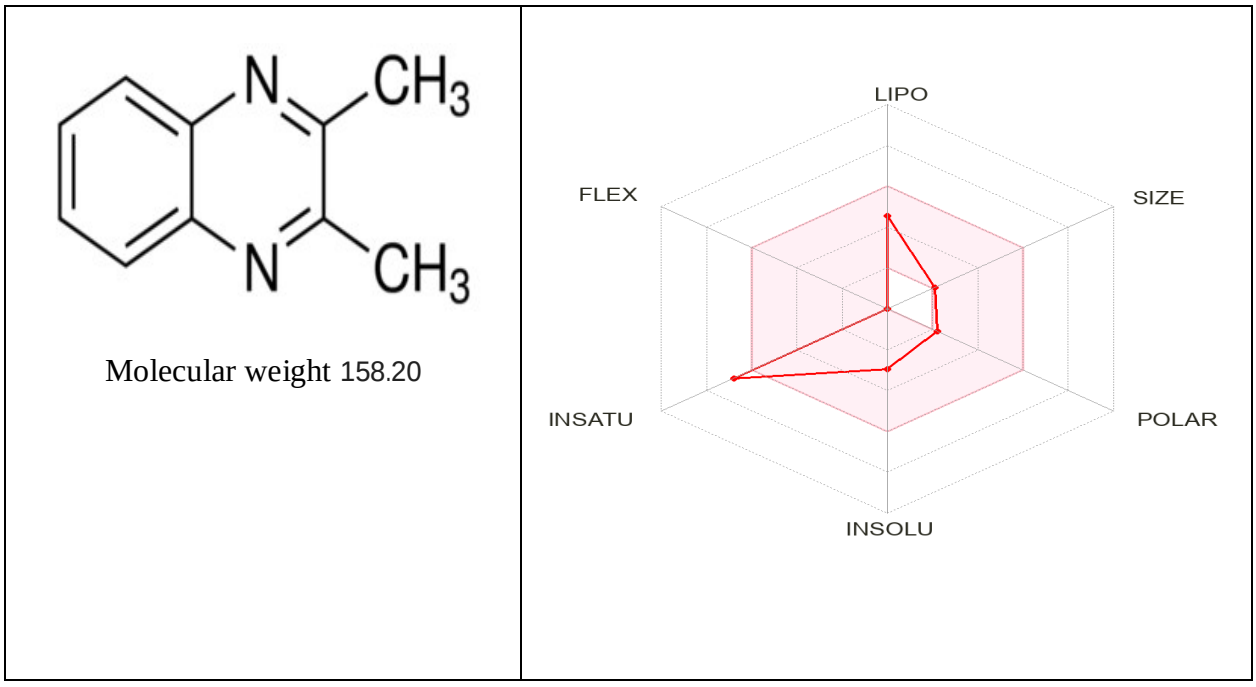


Fig 1: Structure of 2,3-Dimethyquoxaline and its predicated Bioavailability Radar

Six physicochemical properties are considered: lipophilicity(LIPO), size, polarity (POLR), solubility (INSOLU), flexibility (FLEX) and saturation. (INSATU). The pink represents the range of values of these 6 characteristics to consider a molecule as drug-like.

Table 1: Predicated pharmacokinetic characteristics of DMQ

Lipophilicity, skin permeation and Bioavailability Score	
Log $P_{o/w}$ (iLOGP)	2.12
Log $P_{o/w}$ (XLOGP3)	1.73
Log $P_{o/w}$ (WLOGP)	2.25
Log $P_{o/w}$ (MLOGP)	1.49
Log $P_{o/w}$ (SILICOS-IT)	2.88
Consensus Log $P_{o/w}$	2.09
Log K_p (skin permeation)	-6.04 cm/s*
Bioavailability Score	0.55
Topological Polar Surface Area (TPSA)	25.78 A^2
Log K_p cm/s of Betamethasone= -7.32, Diclofenac acid= -4.98 Ketoconazole= -6.46	

Physicochemical stability of DMQ hydrogel

The prepared 1 % DMQ hydrogel was physiochemically stable for 12 months at room temperature (25 ^{o}C in a transparent glass container).

In vivo assessment

Wound contraction % was about 97 % after 7 days after treatment with DMQ 1 % hydrogel compared to about 84 % in the case of the plain hydrogel. Macroscopic wound closure at 0, 7 days after treatment with plain and DMQ 1 % Hydrogel is presented in fig 2. Almost complete wound healing was observed after treatment with DMQ 1 % Hydrogel. Treatment with plain hydrogel was associated with a reduction in wound size but incomplete healing.

Micrographs of H&E-stained wounded tissues of Wister rats are presented in figure 3. The histological study confirmed that treatment with DMQ 1 % hydrogel for 7 days lead to marked attenuation of wound-induced histological changes of the skin documented as the resolution of neutrophile infiltration.

A panel of biochemical markers of oxidative stress markers in skin tissue homogenate is presented in table 2. These data show a slight decrease in GSH and an increase in SOD levels after treatment with DMQ 1 % hydrogel but the level of CAT & MDA are similar to those after treatment with plain hydrogel.

A panel of inflammatory markers in skin tissue homogenate is presented in Table 3: There was a marked reduction of the level of all tested parameters (TNF-α, IL-6 IL-β1 and NF-κB.)

after treatment with DMQ 1 % Hydrogel, These data suggest a potential anti-inflammatory effect of DMQ. Hydroxyproline and TGFB1 in skin tissue homogenate are presented in Table 4. The level of both Hydroxyproline and TGFB1 was increased after treatment with DMQ 1 % Hydrogel suggesting an acceleration of wound proliferation.

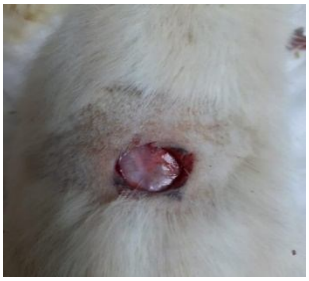
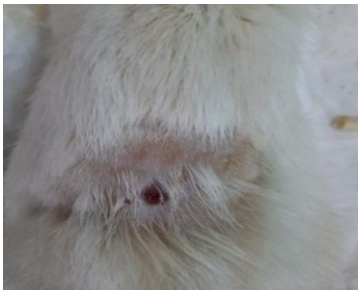
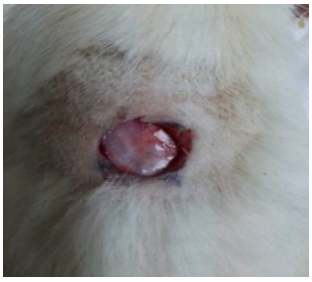
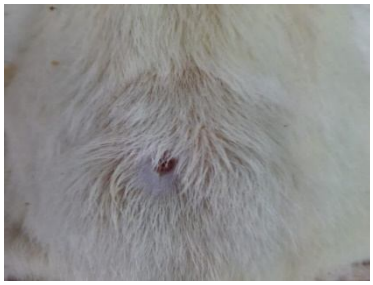
	D0	D7
Plain hydrogel		
DMQ 1% hydrogel		

Fig 2: Wound healing evaluation in Wistar albino rats with complete thickness excision wounds. Macroscopic wound closure at 0, 7 days after treatment with plain and DMQ 1 % Hydrogel

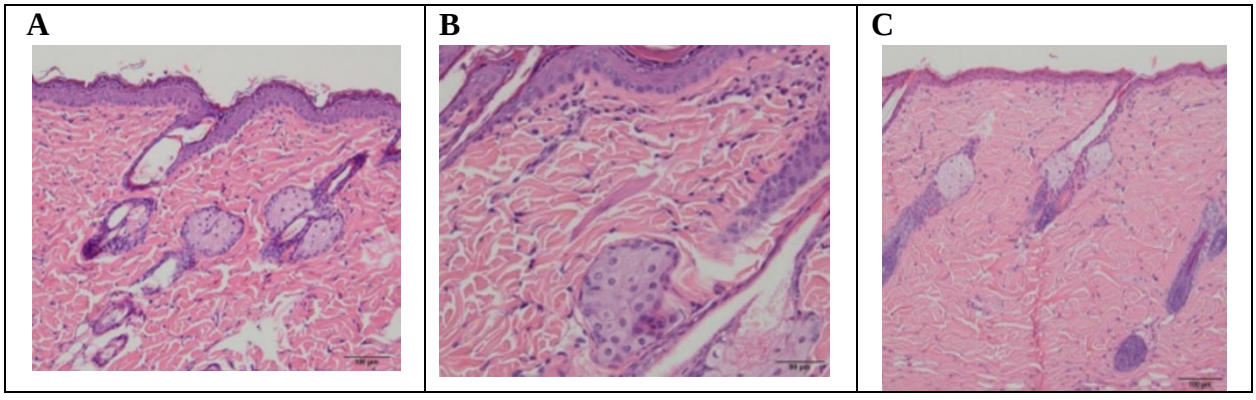


Fig 3: Micrographs of H&E-stained wounded tissues of Wistar rats

A: control (healthy animal) , B: plain gel treated, C: 1 % DMQ gel treated. Skin samples were taken after 7 days of treatment.

Table 2: Panel of biochemical markers of oxidative stress markers in skin tissue homogenate

groups	SOD	CAT	GSH	MDA
	u\ml	u\ml	ng\ml	nmol\ml
DMQ 1%	68	122	2.9	2.00
Plain gel	80	121	4.0	1.98

Table 3: Panel of inflammatory markers in skin tissue homogenate

groups	TNF-α	IL-6	IL-β1	NF-kB
	pg\ml	pg\ml	pg\ml	ng\ml
DMQ 1%	56	16.3	38	44
Plain gel	82	18	56.2	67

Table 4: Hydroxyproline and TGFB1 in skin tissue homogenate in skin tissue homogenate

groups	hydroxyproline	TGF ß1
	pg/ml	pg/ml
DMQ 1%	1.5	10.4
Plain gel	0.94	7.8

Discussion

Computer-aided drug design (CADD) methodologies are increasingly important in drug discovery, particularly in the cost-effective identification of potential drug candidates. These computational techniques are useful for limiting the use of animal models in pharmacological research, assisting in the rational design of innovative and safe drug candidates, repositioning marketed drugs, as well as assisting medicinal chemists and pharmacologists during the drug discovery process) Brogi, Ramalho et al. 2020(. In the present study, ADME prediction revealed that DMQ has moderate water solubility, and excellent lipophilicity.

Target prediction revealed that DMQ is likely to target Family A G protein-coupled receptor, (C-C chemokine receptor type 1, 5 & 8) and protease (Methionine aminopeptidase 1) with high probability. Interestingly chemokines were suggested to play an important role in wound repair. They play a role in the promotion and inhibition of angiogenesis, as well as the recruitment of inflammatory cells that produce growth factors and cytokines to aid in wound healing.(Ridiandries, Tan et al. 2018) . Moreover, DMQ antifungal properties (Alfadil, Alsamhan et al. 2021), and the lack of skin irritation of its topical formulation(Alsamhan 2022) were documented in our previous publications. It was established that 2,3-dimethylquinoxaline form a salt with p-toluenesulfonic acid. It was tested for pharmacology, including Calf thymus DNA binding/cleavage and antioxidant effects. The findings suggested that the compound could interact with DNA via intercalation and should have a weak to modest potential antioxidant effect . The compound also demonstrated antimicrobial and antifungal action.(Murugesan, Saravanabhavan et al. 2014)

All the aforementioned features of DMQ provided the rational that encouraged us to conduct this pilot study to test its efficacy for promoting wound healing.

Because of its exceptional biocompatibility, high moisture resistance, and ability to activate immune cells to speed wound healing, the hydrogel wound dressing is a perfect wound treatment. (Xiang, Shen et al. 2020). In the present study, DMQ was successfully prepared as medicated hydrogel using HPMC. The formulation showed excellent physicochemical stability.

Wound healing is an optimized biological mechanism that occurs in a series of contiguous stages that include hemostasis, inflammatory scenarios, the proliferation of cells, and tissue remodelling.(Goldberg and Diegelmann 2017, Wallace, Basehore et al. 2017).

In the present study, topical treatment of the skin wound led to a slight reduction in SOD level at day 7. However, other oxidative stress markers were insignificantly affected. These observations are likely due to the limitation of the study design in this pilot study. An early sampling of skin tissues e.g., after 3 days is recommended in a future well-designed study. It has been established that reactive oxygen species (ROS) play an important part in several stages of healing. ROS are fundamental to all wound healing processes because low ROS production levels are required to fight against invading microorganisms and cell survival signalling. However, excessive ROS generation or impaired ROS detoxification causes oxidative damage, which is the primary cause of chronic wounds that do not resolve. In this context, experimental and clinical research has shown that antioxidant and anti-inflammatory strategies can help to speed up wound healing.(Cano Sanchez, Lancel et al. 2018). As a result, various antioxidant approaches help improve wound healing(Fitzmaurice, Sivamani et al. 2011, Xu, Han et al. 2020, Comino-Sanz, López-Franco et al. 2021).

The present study showed a moderate reduction of IL-6, but a marked lowering of other biomarkers TNF-α, IL-β1 and NF-κB in the skin tissues of rats treated with DMQ hydrogel compared to the corresponding values in rats treated with plain Hydrogel. Inflammation is a physiological process in the healing process. (Bryan, Ahswin et al. 2012). However, prolonged amounts of ROS production in the wound can lead to chronic inflammation which unfavorably affects wound healing. The inflammation begins with the neutrophil influx, which is aided by mast cell histamine production. The neutrophils promote the release of pro-inflammatory cytokines (e.g., TNF- α, IL-1, and IL-6), phagocytosis, and protease secretion, which contribute to the attraction of other inflammatory cells, the amplification of the inflammatory response, the death of microbial pathogens, and the stimulation of regenerative and remodelling factors.(Rodrigues, Kosaric et al. 2019).

Interleukin- 6 is a proinflammatory cytokine essential for wound healing and plays an important part in acute inflammation. It is released early in reaction to injury and induces the release of proinflammatory cytokines from tissue-resident macrophages, keratinocytes, endothelial cells, and stromal cells. (Johnson, Stevenson et al. 2020).

In the present pilot study, an increase in tissue content of hydroxyproline, and TGF B1 was noticed after treatment with DMQ hydrogel. Hydroxyproline, a non-essential amino acid, is necessary to produce collagen and the thermodynamic equilibrium of collagen's triple-helical

conformation and associated tissues. Its low amount indicates poor wound healing.(Kumar Srivastava, Khare et al. 2016). Transforming growth factor-beta 1 (TGF-β 1) is a multifunctional cytokine implicated in a variety of human illnesses. It is thought to play an essential role in wound healing and repair because it is a key regulator of extracellular matrix production and remodelling via its impact on mesenchymal cells. (Varga, Rosenbloom et al. 1987, Klass, Grobbelaar et al. 2009, Pakyari, Farrokhi et al. 2013).

Several pharmacological approaches for the management of wounds target the inflammatory response (Shukla, Sharma et al. 2019). Certain phytochemicals and natural products can modulate inflammation through a variety of pathways, including the regulation of growth factors and cytokines. Examples of topical application of natural compounds for enhancing wound healing include aloe vera(Hashemi, Madani et al. 2015, Hekmatpou, Mehrabi et al. 2019), It has a broad spectrum of effects that synergistically enhance wound healing. One of these effects is its capacity to suppress inflammatory responses by inhibiting IL-6 and IL-8, reducing leukocyte adhesion, increasing IL-10 levels, and decreasing TNF- α levels.(Mosayebi, Ghazavi et al. 2009). Another example is curcumin, which decreases TNF- α, IL-1, and MMP-9 expression while increasing anti-inflammatory cytokine IL-10 and antioxidant enzymes at the lesion site.(Barchitta, Maugeri et al. 2019).

Conclusion:

DMQ has potential skin wound healing ability likely due to its anti-inflammatory mechanism. Further study is needed to confirm these preliminary findings and to explore the molecular mechanism.

Ethical Approval:

Animal Ethic committee approval has been collected and preserved by the author(s)

Funding: This research work was funded by GENERAL PROGRAMME under grant no. (G235-1440-1441). The authors gratefully acknowledge the technical and financial support provided by 692 the Ministry of Education and King Abdulaziz University, DSR, Jeddah, Saudi Arabia.

Acknowledgements: This research work was funded by GENERAL PROGRAMME under grant no. (G235-1440-1441). The authors gratefully acknowledge the technical and financial support provided by the Ministry of Education and King Abdulaziz University, DSR, Jeddah, Saudi Arabia.

The authors acknowledge prof Soad Shaker Ali, Merit University, Faculty of Medicine, Egypt for her valuable support to discuss the histological finding. The authors acknowledge Dr Shahid Kareem Associate prof of pharmacology Dept of Pharmacology, Faculty of Medicine, KAU for his valuable editing of the manuscript and In Silico Prediction study

Conflicts of Interest: All authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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