




Effect of Flaxseed on TGF-B, IL-6, and MMP9 Genes Expression during Wound Healing Process in Rabbits

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Abstract

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BACKGROUND: Wound healing is a natural restorative response to tissue injury, and it involves regulated order of cellular and biochemical actions to reinstate tissue after injury, which involves resurfacing reconstitution, and restoration of tensile strength of injured skin. Normal and impaired wound healing post-significant problems related to healthcare and expenditure. Most of the chemical medications which widely used for wound healing might cause unwanted side effects with prolonged use such as hyper scarring, thus studies using natural products are now deemed important. Flaxseed is a natural product that enhances the immune system functioning against different diseases. Nevertheless, limited studies have been done looking into the response triggered by immune cells and the wound-healing-related genes with the use of flaxseed extract onto the wounded skin for the healing process.

AIM: The main objective of this study is to analyze the expression of wound healing-related genes during different stages of the wound healing process induced by flaxseed *in vivo*.

METHODS: The effect of flaxseed oil in the early stages (day 4 and 7) and late stages (day 14) of wound healing was explored on New Zealand white rabbits by creating a longitudinal full thickness wound on their back. The gene expression profiles of transforming growth factor-beta (TGF- β), IL-6, and metalloproteinase (MMP9) genes which have roles in wound healing through inflammation, proliferation, and remodeling were studied by polymerase chain reaction method.

RESULTS: Flaxseed extract has significant effects in up-regulating anti-inflammatory marker TGF- β in wounds. Flaxseed oil also reduces the expression level of MMP9 on day 14 of wound healing.

CONCLUSIONS: This suggests that flaxseed extract has the potential to promote wound healing through the regulation of TGF- β and MMP9 *in vivo*.

Introduction

A chronic wound is a significant public health concern that takes up resources from the health-care system globally [1]. The incidence of the chronic wound has been developing in a fashion similar to the “silent epidemic.” In general, wound resulted in a series of social, physical, and psychosocial impact which give rise to social-economic burden. The current health-care system approaches to wound management are always to accelerate wound healing to reduce the social-economic burden while lowering morbidity and mortality [2]. Most of the effort in developing advanced preventive and therapeutic technology in aiding wound healing will benefit higher income countries. Low- and middle-income countries will have lesser accessibility to the advancement of quality healthcare and this remains a challenge [3]. Hence, this work focuses on accessing the wound healing potential of a popularly available oil: flaxseed oil.

Cutaneous or skin wounds are injuries to the outermost protective barrier in which partial or full-thickness skin tissue is lost [4]. This disruption of tissue integrity arises from various causes such as surgeries, traumas, burns, or arterial diseases and can result in either acute or chronic wounds. Wounds can compromise an individual's independence, working capacity, and self-image, which may eventually affect one's quality of life [5]. Therefore, appropriate wound management is critical to achieving optimum healing of a wound. Wound healing process is essentially a series of events that attempts to restore the injured tissue to a normal state, thus avoiding serious complications [6]. It is one of the most complex biological processes in multicellular organisms and can be subdivided into four stages: Hemostasis, inflammation, proliferation, and remodeling. Growth factors, cytokines, and chemokines play a key role in the signaling mechanisms to coordinate the healing process [7]. The activation of cellular proliferation is crucial in the tissue repair and regeneration stage. This wound healing process is not only complex with diverse

cellular and biochemical responses, but it is also fragile and susceptible to interruption or failure leading to the formation of chronic non-healing wounds [8]. In different stages of wound healing, multiple biomarkers work in the orchestra to facilitate the process and advance the healing to the next stage. For example, anti-inflammatory markers such as Transforming growth factor-beta (TGF- β) aid to reduce inflammation and advancing the wound to the proliferation stage [9]. In the first part of our study, we aimed to explore the molecular effect of flaxseed oil on wound healing.

Flaxseed plant (*Linum usitatissimum*) is a member of Linaceae family, originally native in West Asia and the Mediterranean [10]. The oil extract from dried and ripened flaxseeds is one of the richest dietary sources of essential fatty acids [11]. The oil extract consists of 73% of polyunsaturated fatty acids such as α -linolenic acid (ALA) omega-3 and omega-6 fatty acids, and 27% of monounsaturated and saturated fatty acids. The omega-3 fatty acid in the flaxseed oil has been shown to influence the pro-inflammatory cytokine production positively at wound sites [12]. In addition, other essential fatty acids including linoleic acid convey essential nutrients which assist in cellular respiration and membrane regeneration [13]. Furthermore, its richness in flavonoids is believed to play an important role in the tissue regeneration phase of wound healing by improving the collagen fibers strength and minimizing cell damage by enhancing DNA synthesis [14]. The popularity and affordable cost of flaxseed oil make it an attractive candidate for studies to benefit middle and lower-income countries [15]. This study aimed to investigate the molecular effect of flaxseed oil on *in vivo* wounds model.

Materials and Methods

Experimental animals

Twenty-seven (27) male white New Zealand rabbits (6–8 \pm 2.2 months old, 3.1–3.6 kg) were obtained from Laboratory Animal Facility and Management. The animals were kept in the Animal House of IIUM under controlled environmental conditions (20 \pm 5°C, 55 \pm 10% humidity) and were allowed access to water and pellets *ad libitum*. The experiment model was approved by the Animal Ethical Committee (IACUC), Research Management Centre of International Islamic University Malaysia under the ethnic number IIUM/504/14/2/IACUC-2017. All the animal handling protocols were performed in accordance with the guidelines issued by the IIUM Ethics Committee. The animals were acclimatized for 10 days before wound incision surgery.

Tissue samples were obtained at the end of 4, 7, and 14 days for RNA extraction. Total RNA was then subjected to reverse transcription- Polymerase

chain reaction (PCR) to detect the expression of wound healing-related genes (IL6, IFN- γ , Metalloproteinase [MMP9], TGF- β , and VEGF). The levels of expression are determined using GAPDH as an internal positive control.

Wound incision surgery and treatment

The rabbits were divided into three groups: Flaxseed, 2% Fucidin, and the negative control. Flaxseed extract was prepared according to standard ethanol extraction protocol and was applied to the wound. Full thickness wounds were made on both sides of the backbone [14].

All surgical procedure was conducted under aseptic technique. The animals were anesthetized and place on the hard top surgical table. A surgical marker was applied as two dots, measuring 18 \pm 2 mm in length and extending down to the panniculus carnosus, of the rabbits to create a longitudinal (incisional) full thickness wound. A sterilized gauze was applied to minimize hemorrhage [16]. Post-operative meloxicam (1 mg/kg) was given intramuscular for 3 days to control post-operative pain. Subsequently, the animals were randomized to three groups, namely: (I) Negative control (n = 9), (II) positive control (n = 9), and (III) flaxseed (n = 9). The wounds were treated topically with (I) normal saline, (II) 0.5 g of 2% Fucidin cream, and (III) 1 mL of flaxseed oil (4 mg/kg) twice daily for 14 days.

Tissue collection

On days 4, 7, and 14 post-wound incision, three animals from each group were sacrificed and skin tissue around the wound was excised. The tissue was then preserved in RNAlater (Sigma Alrich, US) at –80 °C before RNA isolation.

RNA isolation

For total RNA extraction from the tissue sample, 30 mg samples (stored at –80°C) were thawed on ice and crushed with mortar and pastel under the stream of liquid nitrogen. The powders of tissue were obtained, and total RNA was extracted using the NucleoSpin® RNA Extraction Kit (Macarel Nagel, Germany). The extracted RNA was resuspended in 60.0 μ L RNase-free water and stored at –80°C.

cDNA conversion

cDNA conversion was conducted using the One Script Hot Reverse Transcriptase kit (ABMgood, Canada). Briefly, 1ng total RNA was mixed with 4 μ L 5x RT buffer, 1 μ L dNTP, 1 μ L One Script Hot RTase, and 1 μ L random primer. The mixture was mixed and

centrifuged for 30 s under 10,000 rpm. The mixture was then incubated for 15 min at 60°C follow by 5 min at 85°C. The synthesized cDNA was stored at -20°C.

PCR

This step started with the preparation of the master mixture in a 0.2 mL tubes in volumes, as illustrated in Table 1, as provided in REDiant 2× PCR Master Mix (Germany):

Table 1: Master mix preparation

Ingredients	11X Master Mix (vol)	1X Mix×1X Mix (vol)
Rediant 2×PCR Master Mix	68.75 µL	6.25 µL
Forward primer (0.125 µM)	4.07 µL	0.37 µL
Reverse primer (0.125 µM)	3.27 µL	0.30 µL
Total	76.09 µL	6.92 µL

The master mixture was mixed thoroughly and 18 µL was dispensed into 0.1 mL tubes for each reaction. Next, 2 µL of the sample (cDNA) was added per reaction. The tubes were capped securely and arranged on a plate before the PCR amplification.

Table 2: List of the primers for reverse transcriptase-PCR. GAPDH is used as internal positive control

Gene	Primer sequence (5'-3')
IL-6	FORWARD: ACC ACG ATC CAC TTC ATC C REVERSE: TGT CCT AAC GCT CAT CTT C
MMP-9	FORWARD: TGC CAG GAG TAC CTG TTC CGC TAT REVERSE: TGC CAG TTG AGG TCA CCC TCG
TGFβ	FORWARD: GTG CGG CAG TGG TTG AGC REVERSE: GGT AGT GAA CCC GTT GAT GTC C

PCR: Polymere chain reaction

The primers used are stated in Tables 2 and 3 that show the PCR cycle conditions. On completion, the amplified products were subjected to agarose gel separation and gel imaging [17].

Table 3: PCR cycling conditions

Step	Temperature (°C)	Time	Cycles
Initial denaturation	95	5 min	1
Denaturation	95	3 min	45
Annealing	Depend on primer	15 min	
Extension	72	30 sec	
Final extension	72	10 min	1
Soak	4	Infinity	

Results and Discussion

Gene expression is the phenotypic manifestation of a gene encompassing transcription and translation processes [18]. It occurs in response to environmental changes, thereby providing insights into the cellular activity under certain circumstances. In this study, the gene expression level was screened through the in-house gel electrophoresis-based PCR method, by which samples' total mRNA content was quantified. The target biomarkers included IL-6, MMP-9, and TGF-β.

Wound healing is essential to restoring damaged tissues. It comprises sequential events with three divided stages: a) Inflammatory reaction, b) cell proliferation, and c) remodeling [19]. In this study, each stage was

represented by the respective sampling period as shown in Figure 1 on day 4, day 7, and day 14, corresponding to the time points defined in the previous study [20].

The vascular inflammatory reaction takes place after the onset of the lesion. In the wound area, several pro-inflammatory cytokines like IL-6 are secreted (by macrophages) and thus induce massive leukocyte infiltration from the bloodstream [21]. These leucocytes play an essential role in fighting infection. For instance, neutrophils destroy any harmful pathogens around the wound site. Tissue debris clearance by macrophages eventually provides a strong signal for the resolution of inflammation [22]. Inflammation is a double-edged sword. It presumably facilitates the progress of the wound repair, but prolonged inflammation can lead to chronic wounds. In this study, the gene expression of IL-6 in rabbits treated with flaxseed extract remained constant compared to those with the negative control (regardless of time point, Figure 2, suggesting that the flaxseed extract could prevent the risk of chronic inflammation. A similar effect was manifested in Fucidin treatment.

A. Qualitative and quantitative results of IL-6 gene expression

TGF-β is a cytokine that regulates angiogenesis, cell proliferation, differentiation, extracellular matrix production, and immune modulation in wound healing. It is released by keratinocytes, platelets, and macrophages. The up-regulated TGF-β accelerates the recruitment of inflammatory cells into the wound site and thus initiating inflammation responses [23]. At the proliferation phase, TGF-β1 is critical for re-epithelialization and angiogenesis. Moreover, it involves in migration of fibroblasts, synthesis of extracellular matrix components, and differentiation of fibroblasts into myofibroblasts during the remodeling phase [24]. Several *in vivo* studies have revealed that impaired wound healing is associated with a reduction of TGF-β expression. Our present work found that flaxseed treatment (against negative control) increased the expression of TGF-β on day 4 (1.7 fold) and day 14 (3.7 fold), suggesting its involvement in the inflammation and remodeling stage, respectively (Figure 3). Such effect was superior to the Fucidin treatment. The TGF-β upregulation on day 14 might promote tissue contraction and epithelial closure at the wounds [25]. Moreover, an improved progression of tissue regeneration, including collagen bundle synthesis, vascular and hair follicle development, and fibroblast proliferation as well as fibroblasts differentiation, has been reported on flaxseed oil-treated rabbits [26].

B. Qualitative and quantitative Results of TGF-β Gene Expression

MMP-9 is a Type IV collagenase found in chronic wounds. MMP-9 is diminished during the remodeling stage [27]. Hence, interpreting the MMP-9

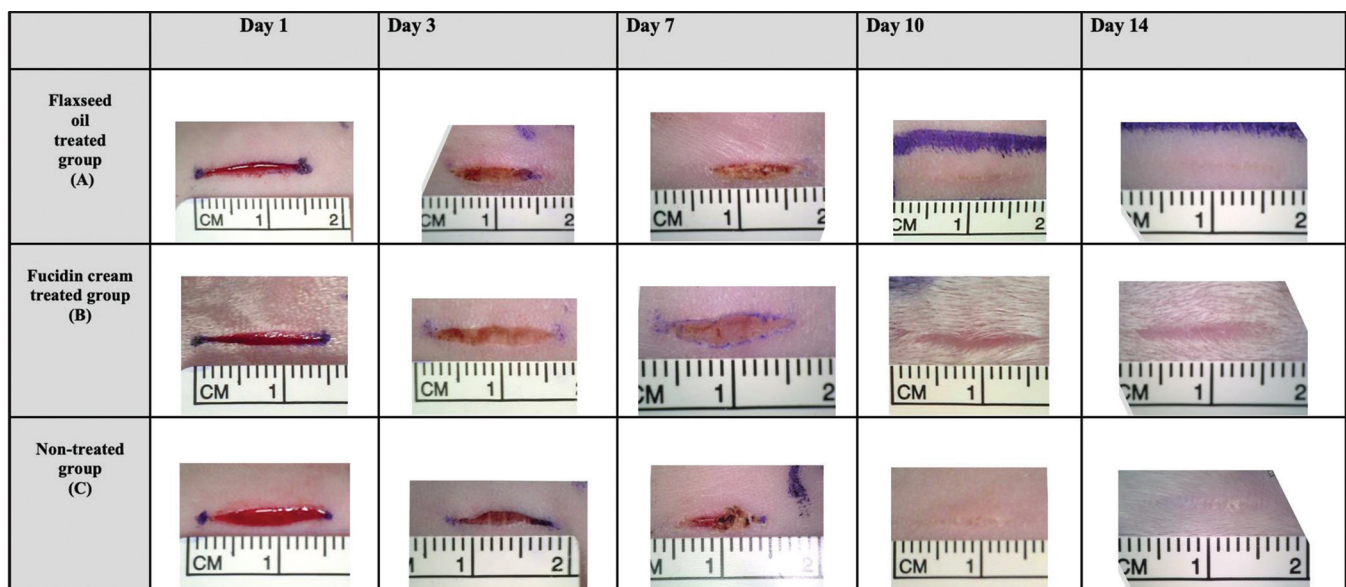


Figure 1: Macroscopic wound healing panorama of NDM groups presenting wound closure length and color changes for the flaxseed, positive control, and negative control groups on respective days

expression level on day 14 reflected its functionality better. As shown in Figure 4, untreated wounds showed a gradual MMP9 accumulation over time. Both fucidin and flaxseed treatments hasten the peak expression on day-7. Meanwhile, on day 14 (vs. negative control), the

fucidin and flaxseed extract treatments caused lower MMP9 expression at six-fold and two-fold degrees,

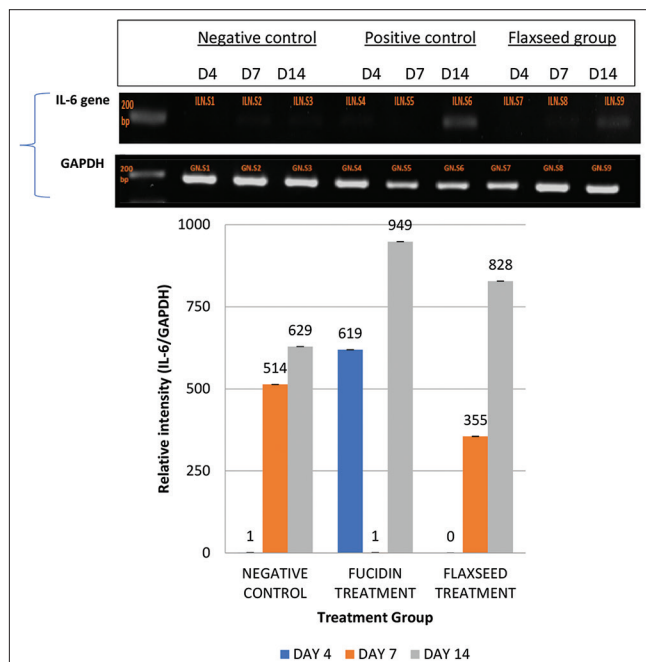


Figure 2: RT-PCR analysis of gene expression for IL-6 at skin wound sites in NDM rabbits. RT-PCR detected the mRNA of these molecules in flaxseed and control skin samples of NDM rabbits. B: The fold change in the baseline gene expression of IL-6 represented the flaxseed influence compared to positive and negative control groups of NDM skin (blue bars represent day 4, orange bars are day 7, and gray bars represent day 14). Data were analyzed using one-way ANOVA. Results were presented as relative intensity \pm standard deviation. The statistical significance was determined at 95% confident interval ($n = 9, p < 0.05$). The comparison was made using the Duncan test as a post hoc test. The analyses were performed in three biological replicates

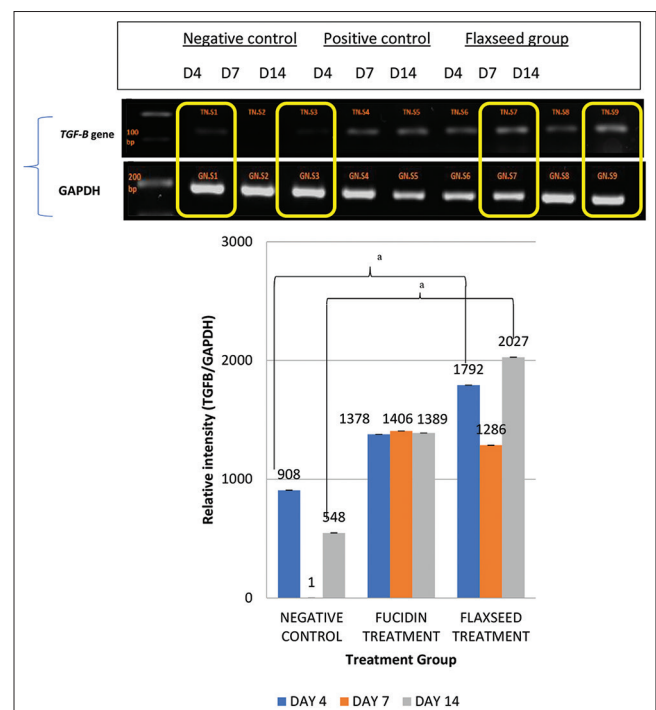


Figure 3: RT-PCR analysis of gene expression for TGF- β at skin wound sites in NDM rabbits. RT-PCR detected the mRNA of these molecules in flaxseed and control skin samples of NDM rabbits. B: The fold change in the baseline gene expression of TGF- β that represented the flaxseed influence compared to positive and negative control groups of NDM skin (blue bars represent day 4, orange bars are day 7, and gray bars represent day 14). Data were analyzed using one-way ANOVA. Results were presented as relative intensity \pm standard deviation. Statistical significance was determined at 95% confident interval ($n = 9, p < 0.05$). The comparison was made using the Duncan test as a post hoc test. The analyses were performed in three biological replicates. ^aSignificant difference ($p < 0.05$) in gene expression level between flaxseed group and untreated negative control group. ^bSignificant difference ($p < 0.05$) in gene expression level between positive control and negative control groups

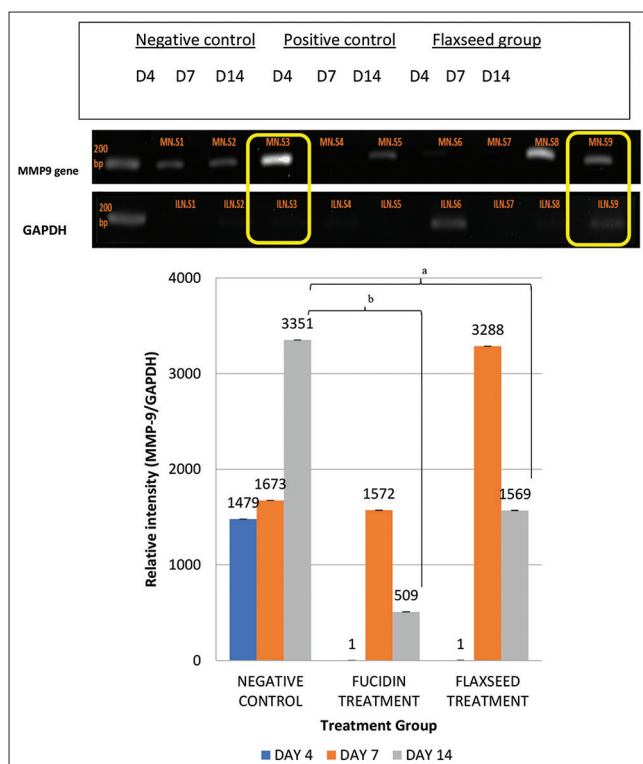


Figure 4: RT-PCR analysis of gene expression for MMP-9 at skin wound sites in NDM rabbits. RT-PCR detected the mRNA of these molecules in flaxseed and control skin samples of NDM rabbits. The fold change in the baseline gene expression of MMP-9 represented the flaxseed influence compared to positive and negative control groups of NDM skin (blue bars represent day 4, red bars are day 7, and green bars represent day 14). Data were analyzed using one-way ANOVA. Results were presented as relative intensity \pm standard deviation. Statistical significance was determined at 95% confident interval ($n = 9$, $p < 0.05$). The comparison was made using the Duncan test as a post hoc test. The analyses were performed in three biological replicates. ^aSignificant difference ($p < 0.05$) in gene expression level between flaxseed and negative control groups. ^bSignificant difference ($p < 0.05$) in gene expression level between positive control and negative control groups

respectively. MMP9 is one of the undesirable markers associated with delayed wound healing. It degrades the extracellular matrix [28], inhibits the keratinocyte from migrating [29], and forms a new attachment to the basal membrane [30]. The MMP9 downregulation indirectly indicated that flaxseed extract could accelerate wound repair without causing prolonged inflammation.

C. Quantitative and qualitative results of MMP-9 gene expression

The Fucidin and flaxseed extract treatments caused lower MMP9 expression at six-fold and two-fold degrees, respectively. MMP9 is one of the undesirable markers associated with delayed wound healing. It degrades the extracellular matrix [28], inhibits the keratinocyte from migrating [29], and forms a new attachment to the basal membrane [30]. The MMP9 downregulation indirectly indicated that flaxseed extract could accelerate wound repair without causing prolonged inflammation.

Conclusions

Flaxseed extract caused changes in the expression level of wound healing-related genes during different phases of the healing process. This suggests the potential role of flaxseed in a wound-healing treatment and highlights a new fundamental knowledge induced by natural product (Flaxseed) which will be the base for introducing the potential of using this plant as one of the possible wound healing medications. This study may have an impact on the future development of naturally based medication for improving the histopathological, immunological, and immunohistochemical response of chronic wound patients; and hence, improved quality of life.

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