Brazilian Journal of Biology®

ISSN 1519-6984 (Print)
ISSN 1678-4375 (Online)

Original Article

Wound-healing and cytokine-modulating potential of medicinal oil formulation comprising leaf extract of *Murraya koenigii* and olive oil

Potencial de cicatrização de feridas e modulação de citocinas de formulação de óleo medicinal compreendendo extrato de folhas de *Murraya koenigii* e azeite de oliva

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Abstract

The study investigated the wound healing effect of medicinal oil (MO) formulation prepared from *Murraya koenigii* leaves extract (methanolic) incorporated in olive oil. The MO was visually transparent, homogenous, smooth in texture, the viscosity grade was observed as 140 cP and easily spreadable. Pro-inflammatory cytokines IL-1β, IL-6, and TNF-α were significantly reduced to 82.3 ± 3.5, 156 ± 6.2, 137.3. ± 5.5 pg/ml, respectively after treatment with MO when compared to disease control animals that showed IL-1β, IL-6, and TNF-α levels of 170 ± 6, 265 ± 7, and 288.6 ± 11, pg/ml respectively. The level of pro-inflammatory cytokine in povidone iodine solution (PIS) group was 95.3 ± 3, 162 ± 6, 177.6 ± 8.9 pg/ml of IL-1β, IL-6, and TNF-α respectively. Interestingly, the wound-healing efficacy of MO was found better as compared to povidone iodine treated standard group and concluded that MO has excellent wound healing effect.

Keywords: medicinal oil, wound healing, *Murraya koenigii*, olive oil, wound model, cytokine network modulation.

Resumo

O estudo investigou o efeito cicatrizante da formulação de óleo medicinal (MO) preparado a partir do extrato de folhas de *Murraya koenigii* (metanol) incorporado ao azeite de oliva. O MO era visualmente transparente, homogêneo, de textura lisa, o grau de viscosidade observado foi de 140 cP e facilmente espalhável. As citocinas pró-inflamatórias IL-1β, IL-6 e TNF-α foram significativamente reduzidas para 82,3 ± 3,5, 156 ± 6,2, 137,3. ± 5,5 pg/ml, respectivamente, após o tratamento com MO quando comparados aos animais controle da doença que apresentaram níveis de IL-1β, IL-6 e TNF-α de 170 ± 6, 265 ± 7 e 288,6 ± 11, pg/ml, respectivamente . O nível de citocina pró-inflamatória no grupo solução de iodopovidona (PIS) foi de 95,3 ± 3, 162 ± 6, 177,6 ± 8,9 pg/ml de IL-1β, IL-6 e TNF-α, respectivamente. Curiosamente, a eficácia de cicatrização de feridas de MO foi encontrada melhor em comparação com o grupo padrão tratado com iodopovidona e concluiu que a preparação de MO tem efeito de cicatrização de feridas.

Palavras-chave: óleo medicinal, cicatrização de feridas, *Murraya koenigii*, azeite, modelo de ferida, modulação da rede de citocinas.

1. Introduction

Wound healing is a series of complex processes that comprise various phases such as reduction of inflammation, epithelialization, angiogenesis, matrix deposition, and remodeling (Moni et al., 2018). Cytokines are involved in the recruitment of fibroblasts and epithelial cells because of inflammatory leukocyte stimulation, which leads to the development of granulation tissue. On the other hand, persistent microbial burden, accumulation of excess inflammatory proteins and biofilm formation are also the limitations associated with drug resistance in treatment of wounds. In addition, the generation of reactive oxygen species (ROS) aggravates

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Received: September 8, 2021 – Accepted: February 13, 2022

inflammatory response and induces impairment of healing of cutaneous wounds.

The development of novel pharmaceutical formulations with immunomodulatory and antibacterial properties for wound healing has a major effect on bacterial colonization of wounds. Traditionally, medicinal plants are vital sources of therapeutic principles for many diseases globally (Moni et al., 2021a; Lin et al., 2020; Rakotoarivelo et al., 2015). *Murraya koenigii* (*M. koenigii*) is an aromatic tree which belongs to the family Rutaceae, popularly known as curry leaf. It is grown in tropical and sub-tropical regions for its high medicinal value and characteristic aroma (Noolu et al., 2013; Vandana et al., 2012). *M. koenigii* is widely used in traditional medicine and home remedies in India, Pakistan, Sri Lanka, China and Africa (Balakrishnan et al., 2020). The leaves of *M. koenigii* have been used medicinally for their anthelminthic, antiemetic, analgesic, digestive, appetite-stimulating, anti-dysentery, anti-pile, anti-inflammatory, and anti-itch properties, and used for healing of cuts, bruises, and oedema (Bhandari, 2012). Globally, chronic skin wounds represent a common health problem because such injuries are related to cut, scrape or scratch the skin (Desai et al., 2012). Chronic skin wounds are a common health problem worldwide.

According to Costa et al. (2016) Olive oil improves the healing of cutaneous wounds in chronically stressed mice due to its anti-inflammatory and antioxidant properties. In our previous study, it has been reported that methanolic extract of the leaves of *M. koenigii* contained steroids, phytosterols, terpenes, fatty acids and furofuran lignan (Moni et al., 2021b) which indicated the presence of immunomodulatory principles as major bioactive components. In continuation of previous research, the current study sought to determine the synergistic wound-healing efficacy of a methanolic extract of *M. koenigii* leaves and commercially available olive oil in a MO formulation through its effect on the cytokine network.

2. Experimental

2.1. Plant collection and processing

M. koenigii stems with leaves were collected from trees in Jazan, the capital city of Jazan province, Kingdom of Saudi Arabia. The stem with leaves was washed twice in tap water at the point of collection and kept in slanting position for a few minutes to drain out the water. The washed specimens were identified in the herbarium of Jazan University (JAZUH), with the reference number 1215 (JAZUH). A voucher specimen of the plant was also deposited at the herbarium of JAZUH for future reference. The samples were packed in polythene bags (biohazard yellow bags), tied, and transported to the laboratory. The leaves were plucked out from the stems of the plant and thoroughly washed with Millipore water to remove any impurities present. The washed leaves were dried under shade for one week. The air-dried samples were cut into small pieces and powdered using a grinder. The finely powdered leaf samples obtained were pooled and stored in an air-tight container prior to use.

2.2. Solvent extraction

200 g powdered sample was packed in Soxhlet apparatus and subjected to methanolic extraction through hot continuous percolation technique at 60 °C for 3 h. Finally obtained solvent containing sample was transferred into glass beaker and evaporated at room temperature (Moni et al., 2021b).

2.3. Formulation of medicinal oil (MO)

Commercially available virgin olive oil (OLIO SASSO, Italy) was purchased from a local market. The clear viscous MO was prepared by constantly agitating the mixture of olive oil and dried extract (50% w/v) at 60 °C using glass stirrer. The oil was stored in a clean, sterilized glass bottle with screw cap at room temperature for further use.

2.4. Determination of viscosity of MO

The viscosity of MO was determined using Brookfield digital viscometer (Model LVDV-E, USA) with spindle S63. A 50 ml MO was transferred in to a 50 ml beaker and then allowed to settle for 5 min. The viscosity was measured at a rotating speed of 30 rpm at room temperature.

2.5. Wound healing studies

2.5.1. Selection, acclimatization and grouping of animals

Healthy male Wistar rats weighing 170 – 200 g were purchased from the Central Animal facility of Jazan University, Jazan. They rats were acclimatized at standard laboratory conditions (22 ± 08 °C, and relative humidity of 56 ± 6%) in institutional animal house of College of Pharmacy, Jazan University. Animals were kept free access to a standard autoclaved laboratory diet and water. Prior to commencement of experimentation, the entire study protocol was approved from Institutional Research Review and Ethics Committee (IRREC- 905/1012/1441).

2.5.2. Experimental design

Animal were divided in to four groups of six animals in each. The group distribution is as follows:

Group 1: Normal control group: The animals of the group were without wounds and therefore did not receive oil treatment.

Group 2: Disease control group: The excision wounds were created on Wistar rats according to the procedure established by Moni et al. (2018). Excision wound model was adapted for the measurement of wound contraction and epithelization in rats. Animals were given light ether anesthesia (diethyl ether) and their dorsal skin was shaved using electrical shaver. A circular piece (10 mm) of full thickened skin was cut off from the pre marked area using sterile biopsy punch. Wound areas were measured and recorded on day 3, 5, 8 and 12 for all groups on a graph paper. Meanwhile animals were also inspected for sign of infection, and infected animals were excluded from the study and replaced. In this group, the animals did not receive any oil treatment.

Group 3: Standard drug treatment group: The animals treated with 100 µl of 10 % w/v povidone iodine solution PIS on excision wound daily twice morning and evening.
Group 4: Treatment with MO: The animals treated with 100 µl of MO on excision wound daily twice morning and evening.

2.5.3. Measurement of wound contraction

Wound size of was measured with a transparent ruler at the outset, and subsequently at 2-day intervals up to day 12. The percentage (%) healing of wound was calculated using the following Formula 1 (Nagar et al., 2016):

$$Wound\ healing\ (\%) = \frac{(Initial\ wound\ area) - (wound\ area\ on\ a\ specific\ day)}{(Initial\ wound\ area)} \times 100 \quad (1)$$

2.5.4. Collection of blood specimens

On sixth day of initiation of wound, animals were anesthetized under light ether anesthesia and blood samples were collected via ocular puncture. Serum samples were separated by centrifuging at 3000 g for 10 min. Then the serum samples were stored at -20 °C for the measurement of cytokine. Sandwich enzyme linked immunosorbent assay (ELISA) kits (Abcam, USA) were used to determine/estimate the serum levels of the proinflammatory cytokines IL-1β, IL-6, and TNF-α as described below.

2.5.5. Measurement of pro-inflammatory cytokines

Serum levels of IL-1β, IL-6 and TNF-α were measured quantitatively using their respective rat ELISA kits (ABCAM, USA). The assays employed a simple step sandwich ELISA to determine the serum level of each cytokine. Standards and samples were simultaneously pipetted and dispensed into their respective ELISA wells and incubated at room temperature for 2.5 h. During the incubation, the cytokine present in the sample was bound to the wells coated with immobilized specific antibodies. After incubation, the wells were washed thoroughly with 1× wash solution using Biotek ELISA washer elx50, USA. Then, the specific 1× biotinylated anti-rat antibody was added and incubated at room temperature for 1 h, with mild shaking. Thereafter, the plates were washed as previously described, to remove unbound biotinylated antibody. Then, HRP-conjugated streptavidin was pipetted out and added to the wells. The wells were washed again using the same washing parameters as described earlier. Then a TMB substrate solution was added to these wells and incubated for 30 min at room temperature in dark with mild shaking. Following this, stopping solution was added, and the intensity of the colour developed was measured by determining its absorbance at 450 nm using a BioTek ELx 800 ELISA reader. The absorbance was directly proportional to the amount of each cytokine bound to its specific antibody.

2.5.6. Statistical analysis

Statistical analysis was performed by using Graph pad Prism software (Version 8.3.1), USA through one-way analysis of variance (ANOVA), followed by Tukey Kramer analysis as a post-hoc test.

3. Results and Discussion

Wound healing is a complex sequential process involving haemostasis, proliferation, vascularization, matrix production and remodeling (Cogo et al., 2021; Reinke and Sorg, 2012). Many types of cells are involved in the wound healing processes such as immune cells, endothelial cells, keratinocytes, and fibroblasts (Valluru et al., 2011; Singer and Clark, 1999). Herbs have significant role in treating various diseases and the leaves of *M. koenigii* have been reported for numerous medicinal properties. Earlier studies have demonstrated that aqueous extract of *M. koenigii* accelerated the wound healing process (Shukla and Kashaw, 2019). It has been reported that methanol extract of the leaves of *M. koenigii* contained bioactive compounds such as epiyangambin, stigmasterol, eucalyptol, ethyl cinnamate, a-terpineol, fatty acids, and steroids (Moni et al., 2021b). The present study was aimed at investigating the wound healing efficacy of medicinal oil (MO) prepared by mixing the methanolic extract of leaves of *M. koenigii* with olive oil, with respect to its effect on levels of proinflammatory cytokines which are critical in the wound healing progress.

In the present study, MO was successfully prepared using a simple triturating method. The viscosity grade of MO was 140 cP, implying that it was visually transparent, homogenous, and smooth-textured. Table 1 depicts the percentages of wound healing in the various treatment groups. During treatment, the effect of MO on wound healing was manifested from the 3rd day onwards. The results showed that MO exhibited excellent wound healing properties as the rats fully recovered before 12 days of the study period. The wound recovery by MO was significantly higher than PIS. This may be due to the synergistic combination of methanolic extract of the leaves of *M. koenigii* and olive oil. In an earlier study, it was reported that olive oil promoted wound healing on excisional wound (Schanuel et al., 2019; Donato-Trancoso et al., 2016). Low kinematic viscosity of oil is essential for the bioavailability of drugs at the tissue level (Rahman et al., 2012). The MO used in this study was viscous but easily

Table 1. The percentage wound healing of various treatment groups.

Day	Group 2	Group 3	Group 4
3	16.6 ± 1.5	26 ± 2$^{\#\#}$	36 ± 1$^{\$\$, ***}$
5	41.3 ± 1.52	44.6 ± 1.5 ^{ns*}	48.6 ± 2$^{ns, \#\#}$
8	58 ± 1	64 ± 1$^{\#\#}$	70.3 ± 2$^{***, \$\$}$
12	73.6 ± 2	91.6 ± 0.5***	98.6 ± 0.5$^{***, \$\$}$

Values are the mean ± SD, n=6, Data were analyzed by Tukey Kramer analysis (Sample vs Control), post hoc test. $^{\#\#}$Significant when compared to Group 2 at $p < 0.01$; $^{\$\$}$Significant when compared to Group 3 at $p < 0.01$; ***Extremely significant when compared to Group 2 at $p < 0.001$; ns non-significant when compared to Group 3 at $p < 0.01$; ^{ns*}Non-significant when compared to Group 2 at $p > 0.05$ Group 2: Disease control (Wound without treatment); Group 3: Standard control (Treatment with povidone iodine solution, PIS); Group 4: Medicinal oil (MO) treatment group.

spreadable at the site of injury. Therefore, the bioactive molecules in MO penetrated the wound tissue, resulting in healing effect. The synergistic healing effect of olive oil and honey on diabetic foot ulcers has been reported in a study by Karimi et al (2019). Olive oil is composed of hydroxytyrosol, oleuropein and many phenolic compounds with antibacterial and antioxidant properties (Moustafa and Atiba 2015; Al-Waili, 2003). Moreover, olive oil contains monounsaturated fatty acids which enhance the immune function (Yaqoob et al., 1998). An earlier study suggested that a methanolic extract of *M. koenigii* dried leaves had a substantial anti-inflammatory and analgesic effect when compared to the conventional medication diclofenac sodium (Gupta et al., 2010). According to Ani et al. (2016) the methanolic extract of *M. koenigii* leaves displayed antinociceptive and analgesic effect. Hydroalcoholic extract of *M. koenigii* fruit possesses substantial anti-inflammatory and wound healing effects (Gummalla et al., 2016).

The levels of pro-inflammatory cytokines in the treatment groups are presented in Table 2. In this study, the levels of pro-inflammatory cytokines IL-1β, IL-6, and TNF-α were increased significantly in disease control animals (Group 2) following creation of the wound. It has been reported that proinflammatory cytokines were elevated by wound creation in streptozotocin-induced diabetes (Moni et al., 2018). The levels of pro-inflammatory cytokines were decreased when the wound treated with MO, when compared with the group treated with PIS. Figure 1 shows the levels of IL-1β in the various treatment groups. Group 2 represents the disease control group in which the IL-1β level was increased 136.4% after creating the wound, when compared to normal healthy rats (normal control, i.e., Group 1). Interestingly, treatment of the wounds with MO (Group 4) markedly reduced the IL-1β level by 51.58%, a level of reduction which was significant, when compared to PIS treatment group ($p < 0.05$). The level of IL-6 (Figure 2), a potent pro-inflammatory cytokine, was reduced significantly by about 48% by MO treatment in group 4, while IL-1β reduction level was significant, relative to that of PIS treatment in group 3 ($p < 0.05$). Moreover, TNF-α, a pro-inflammatory marker was reduced when the wounds were treated with MO (Group 4), when compared to PIS treatment group (Group 3; $p < 0.01$). Figure 3 shows the serum levels of TNF-α in the various treatment groups. Serum TNF-α was reduced by about 50%, when compared to the disease control group (Group 2). The percentage reductions in the levels of pro-inflammatory cytokines were in the order: IL-1β > TNF-α > IL-6, although the reduction levels were more or less equal. Figure 4 shows stepwise *in vivo* wound-healing effects of MO and PIS. It was observed that MO had better wound-healing property than PIS.

Wound-infiltrating macrophages, dendritic cells, keratinocytes, fibroblasts, and mast cells release IL-1β, a pro-inflammatory cytokine which exerts pleiotropic mode of action. IL-6 is also a pleiotropic cytokine involved in tissue injuries, chronic inflammation, autoimmunity, and bacterial infection of wounds. It is released from tissue-resident macrophages, keratinocytes, endothelial cells, and stromal cells (Li et al., 2020; Rose-John et al., 2017). IL-6 is also associated with induction of chemotaxis of leukocytes in the wound and modulates immune response during the healing process by regulating leukocyte infiltration, angiogenesis, and collagen accumulation (Ambrosch et al., 2008).

TNF-α is a cytokine derived from macrophages in the effector phases of inflammatory reactions, and it serves as an inflammatory biomarker. It plays a critical role in inflammatory response and promotes wound healing by enhancing wound epithelialization and neovascularization

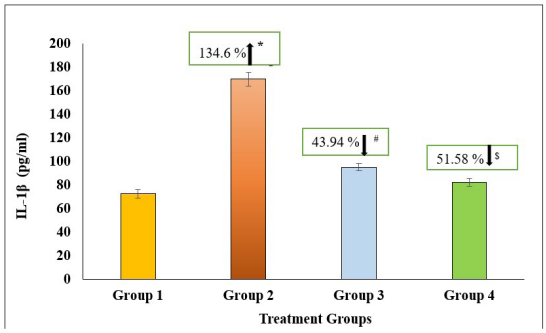

Figure 1. Serum IL-1β level of treatment groups. *The level of IL-1β enhanced 134.6% when compared to Group 1; # The level of IL-1β declined 43.9% when compared to Group 2; $The level of IL-1β declined 51.8% when compared to Group 2.

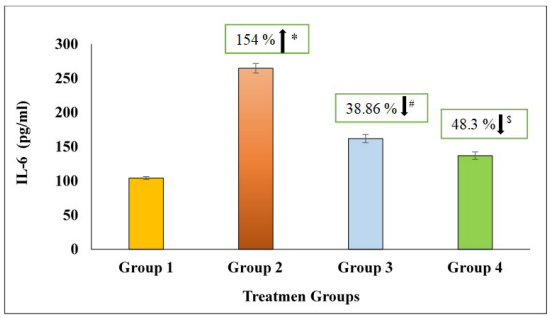

Figure 2. Serum IL-6 level of treatment groups. *The level of IL-6 enhanced 154% when compared to Group 1; #The level of IL-6 declined 38.86% when compared to Group 2; $The level of IL-1β declined 48.3% when compared to Group 2.

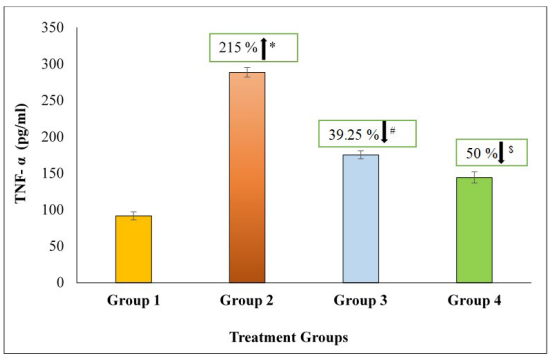

Figure 3. Serum TNF- α level of treatment groups. *The level of TNF- α enhanced 215% when compared to Group 1; #The level of TNF- α declined 39.25% when compared to Group 2; $The level of IL-1β declined 50% when compared to Group 2.

Table 2. Pro-inflammatory cytokines in treatment groups.

Treatment groups	Pro-Inflammatory cytokines levels		
	IL-1β	IL-6	TNF-α
	(pg/ml)	(pg/ml)	(pg/ml)
Group 1	72.6 ± 3.7	104.3 ± 2	91.6 ± 5.5
Group 2	170 ± 6	265 ± 7	288.6 ± 11
Group 3	95.3 ± 3***	162 ± 6***	177.6 ± 8.9***
Group 4	82.3 ± 3.5 ns, **	156 ± 6.2 ns, **	137.3. ± 5.5 $$, ###

Values are the mean ± SD, n=6, Data were analyzed by Tukey Kramer analysis (Sample vs Control), post hoc test. ns: non-significant when compared to Group 1 at p >0.05 level. **Significant when compared to Group 3 at p <0.05 level; $$Significant when compared to Group 3 at p < 0.01 level; ###Extremely significant when compared to Group 2 at p < 0.001 level; ***Extremely significant when compared to Group 2 at p < 0.001 level. Group 1: Normal control group; Group 2: Disease control (Wound without treatment); Group 3: Standard control (Treatment with povidone iodine solution, PIS); Group 4: Medicinal oil (MO) treatment group.

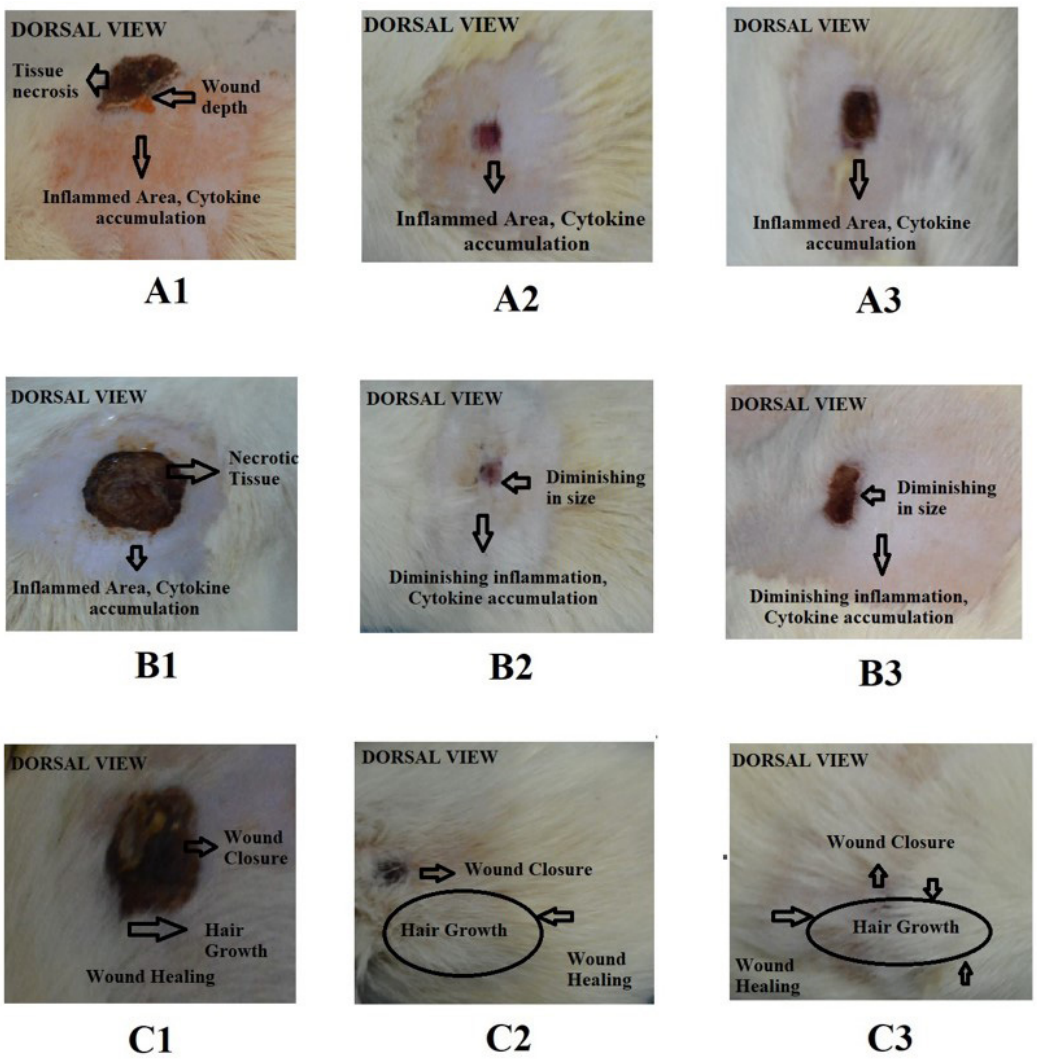

Figure 4. A comparative wound healing study. (A1) Control animals, Dorsal view of wound on the 3rd day after creating wound; (A2) Dorsal view of the wound after treating with povidone-iodine solution (PIS) on 3rd day; (A3) Dorsal view of the wound after treating with medicinal oil (MO) on 3rd day; (B1) Control animals, Dorsal view of wound on the 6th day after creating wound; (B2) Dorsal view of the wound after treating with povidone-iodine solution (PIS) on 6th day; (B3) Dorsal view of the wound after treating with medicinal oil (MO) on 6th day; (C1) Control animals, Dorsal view of wound on the 9th day after creating wound; (C2) Dorsal view of the wound after treating with povidone-iodine solution (PIS) on 9th day; (C3) Dorsal view of the wound after treating with medicinal oil (MO)on 9th day.

(Johnson et al., 2020; Ritsu et al., 2017; Frank et al., 2013). The present study showed that MO reversed wound-induced significant increases in the levels of IL-1β, IL-6, and TNF-α and the wound-healing efficacy of MO was significantly higher than that of PIS.

4. Conclusion

The results of present study indicated that MO has effective *in vivo* wound-healing property. MO also effectively modulated the immune system through downregulating the expressions of pro-inflammatory cytokines and may be the contributing factor in wound healing. However, further investigation is required to get more insight.

Acknowledgements

The authors acknowledging Bio render, Toronto, Canada for creating graphical abstract.

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