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Karo Traditional Oil, a traditional herbal medicine from Indonesia promote wound healing acceleration by suppressing tumor necrosis factor - α and stimulating interleukin 10 production

El Aceite de Karo Tradicional, una medicina herbaria tradicional de Indonesia, promueve la aceleración de la curación de las heridas al suprimir el factor de necrosis tumoral - α y estimular la producción de interleucina 10

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SUMMARY

Introduction: *Wound healing acceleration is often found on open wounds smeared with Karo Traditional Oil, but its mechanism of action is unknown. Therefore, this study aimed to assess the mechanism of action of active compounds in Karo Traditional Oil in the inflammation phase of the wound healing process.*
Methods: *A randomized post-test-only control group design study was conducted on 54 male Wistar rats. The negative control was treated with NaCl moist gauze. The positive control was treated with Karo Traditional Oil's carrier oil gauze, the treatment group was treated with Karo Traditional Oil gauze and observed on the first, third, and seventh day.*
Results: *There was a significant increase in inflammatory cells on 1st-day post-treatment in the treatment group compared to the negative and positive control groups (p= 0.001). There was a significant decrease in Tumor Necrosis Factor - α (TNF- α) cytokines level on the first day (p= 0.002) and day-7 (p= 0.007). There was a significant decrease in Interleukin (IL)-10 cytokine level on the first day (p= 0.012), then a significant increase on the seventh day (p= 0.002).*
Conclusion: *Active compounds in Karo Traditional Oil acts as a potent inflammation regulator in the wound healing process by suppressing the pro-inflammatory cytokines and promoting anti-inflammatory cytokines.*

Keywords: *Karo Traditional Oil, wound healing, TNF-α, IL-10, inflammation cells.*

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RESUMEN

Introducción: *La aceleración de la cicatrización de heridas se ha observado a menudo en heridas abiertas untadas con el Aceite Karo Tradicional, pero su mecanismo de acción se desconoce. Por lo tanto, este estudio tuvo como objetivo evaluar el mecanismo*

de acción de los compuestos activos en el Aceite Karo Tradicional en la fase de inflamación del proceso de cicatrización de heridas.
Métodos: *Se llevó a cabo un estudio de diseño de grupo de control aleatorizado posterior a la prueba en 54 ratas Wistar macho. El control negativo se trató con una gasa húmeda de NaCl. El control positivo se trató con una gasa con el vehículo del Aceite Karo Tradicional, el grupo de tratamiento se trató con gasa con el Aceite Karo Tradicional y se observó en el primer, tercer y séptimo día.*
Resultados: *Hubo un aumento significativo de células inflamatorias en el primer día después del tratamiento en el grupo de tratamiento en comparación con los grupos de control negativo y positivo (p= 0,001). Hubo una disminución significativa en el nivel de citocinas como el factor de necrosis tumoral - α (TNF-α) el primer día (p= 0,002) y el día 7 (p= 0,007). Hubo una disminución significativa en el nivel de la citocina interleucina (IL) -10 el primer día (p= 0,012), la cual aumentó significativamente el séptimo día (p= 0,002).*
Conclusión: *Los compuestos activos en el Aceite Karo Tradicional actúan como un potente regulador de la inflamación en el proceso de curación de heridas al suprimir las citocinas proinflamatorias y promover las citocinas antiinflamatorias.*

Palabras clave: *Aceite Karo Tradicional, cicatrización de heridas, TNF-α, IL-10, células de inflamación.*

INTRODUCTION

Nowadays, wound healing is still an important issue among scientists, practitioners, and clinicians because as time goes by, the demands of society for wound healing and its management to achieve the maximum possible results are also higher. Both acute and chronic wounds require good wound care and management. The series of activities include cleaning the wound and changing the dressing (1). A study in Surabaya, Indonesia, shows that modern dressing in healing was better compared to classic dressing (2). One of the ultimate goals of wound healing is to speed up the process of re-epithelialization by controlling the inflammation phase.

The more complex wound problem can be seen from the increasing number of incidents of injuries every year. According to a study, there were 20 million acute traumatic wounds and lacerations (3). Recent research in the United States shows the prevalence of patients with injuries is 3.50 per 1 000 population, with the most significant cause of injury to the world's population is surgery/trauma injuries (48.00 %) (3). American Wound Association researched the incidence of wounds in the world based on the etiology of the disease. The most acute wound data were obtained: 110 300 000 surgical cases, 20 400 000 abrasions, 10 000 000 burns, and 1 600 000 trauma injuries (4). In Indonesia, the infection rate for surgical wounds reached 2.30, up to 18.30 % in 2001, and is currently increasing to 55.1 %. This phenomenon indicates the increasing number of wound healing complications that cause prolonged inflammatory processes, and the wound healing time cannot be estimated (5,6).

Open wounds, which take a long time to heal, are usually caused by the prolonged inflammation phase. A report stated that the application of cellulose is better than zinc oxide non-eugenol on the healing of open wounds after periodontal surgery (7). However, the longer the wound heals, the more susceptible it is to microorganisms exposure and the higher infection risk and complications of other diseases it will have. Increase treatment costs and are prone to cause functional and aesthetic problems such as the appearance of contractures, keloids, and hypertrophic scars (1,4,8).

Pharmacologically, the antiseptic drug that is often used for wound healing today is Povidone-iodine (9). However, povidone-iodine has a less significant effect on decreasing bacterial colonization in contaminated wounds, and some systemic side effects such as skin hypersensitivity reactions, swelling on the face also cause anxiety, depression, and myxoedema. Povidone Iodine is also corrosive and can damage fibroblast tissue. While the application of soft silicon dressing helps treat pain in wounds, it cannot accelerate wound healing, and the costs are quite expensive (10,11).

Experimental research concludes that Sukun leaf extract effectively increases collagen density in the healing process of excision wounds (12). A review stated that curcumin-loaded chitosan nanoparticle has the potential to accelerate the post-extraction wound healing in Diabetes Mellitus (DM) patients by decreasing Reactive Oxygen Species (ROS) levels in all stages of wound healing (13). A study also reveals that 10 % of standardized pomegranate extract

accelerates the healing of deep second-degree burn wounds (14). An empirical data on the use of Karo Traditional Oil shows that 42.86 % are wound treatments, and 99.3 % of them have been declared cured with results as expected. Components of the majority of Karo Traditional Oils consist of bicyclic monoterpene compounds with the most composition, 74.47 % composed of α-pinene compounds, which are known to have potent inflammatory regulatory effects so that they can accelerate the wound healing process phase (15). Therefore, this study aims to observe the mechanism of action of active compounds in Karo Traditional Oil in the inflammation phase of the wound healing process.

METHODS

Randomized post-test only control group design research on fifty-four male Wistar rats weighing 150 ± 30 g from Gajah Mada University Integrated Testing Research Institute Jogjakarta, Indonesia, was housed in an animal unit 23 oC least two weeks before the experiments. The rats were housed in individual cages, free access to water and food pellets, and divided randomly into nine groups.

The rat was injected with a 0.3 mL/100 x (weight in gram) Rat cocktail, consisting of Ketamin 2 mL, Xylazine 1.25 mL, ACP 0.33 mL, and NaCl 6.41 mL intramuscular. Square full-thickness wound was created on the right back of each rat with 2.5 x 2.5 cm size, and the negative control group was treated with Natrium Chloride (NaCl) moist gauze compresses, the positive control group treated with the gauze compresses of Karo Traditional Oil's carrier oil, treatment group treated with Karo Traditional Oil gauze compresses and observed on the first day, the third day, and the seventh day. The same person did all the procedures to minimize differences in the force of the person applies.

After the 7th day, all the rats were sacrificed, and the wound with surrounding tissues was separated and fixed in 10 % buffered formalin for 24 hours at room temperature. After fixation and dehydrated in grade ethanol, cleared in xylene, and embedded in paraffin, the sections were mounted in glass slides and stained with hematoxylin and eosin. Two pathologists inspected all slides without information to earlier treatment under magnifying instruments from x40 to x100 amplifications. The number of inflammatory cells counted by histopathological examination of Histological Examination (HE) painting, cytokine levels of Tumor Necrosis Factor- α (TNF- α) and Interleukin (IL) -10 counted by immunohistochemical examination.

Data were analyzed using ANOVA statistical test, followed by a post hoc multiple comparisons with SPSS 23 software, and are expressed as mean ± Standard Deviation (SD). The data were tested for normality and homogeneity. Statistical significance was accepted at $p < 0.05$.

RESULTS

Table 1 and Figure 1 show a significant increase in inflammatory cells on the first day post-treatment in the treatment group compared to the negative and positive control groups ($p = 0.001$).

Table 1

Inflammation Cells Count Analysis of the Studied Groups

Groups	First day Mean ± SD	Third day Mean ± SD	Seventh-day Mean ± SD
Negative control	0.47 ± 0.48 [a]	2.10 ± 0.68	157 ± 0.79
Positive control	1.13 ± 0.47 [b]	2.00 ± 0.93	2.07 ± 0.80
Treatment group	1.83 ± 0.56 [c]	2.00 ± 0.52	2.13 ± 1.00
P value	0.001*	0.786	0.484

* Statistically significant difference between a, b, and c
SD = Standard Deviation

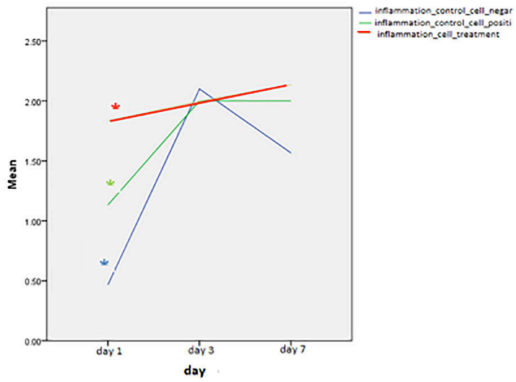

Figure 1. Inflammation cells count comparison graphic between the studied groups.

Table 2 and Figure 2 show a significant decrease in TNF-α cytokines level on the day-1 and day-7 day ($p=0.002$; $p=0.007$; respectively).

Table 2

TNF- α Cytokine level count analysis of the studied group

Groups	First day Mean ± SD	Third day Mean ± SD	Seventh-day Mean ± SD
Negative control	3.40 ± 1.18 [a]	1.50 ± 0.60	1.90 ± 0.41 [a]
Positive control	2.90 ± 0.84 [a]	1.53 ± 0.16	3.43 ± 1.47 [a]
Treatment group	1.33 ± 0.43 [b]	1.07 ± 0.53	0.90 ± 0.37 [b]
P value	0.002*	0.197	0.007*

*Statistically significant difference between [a] and [b]
SD = Standard Deviation

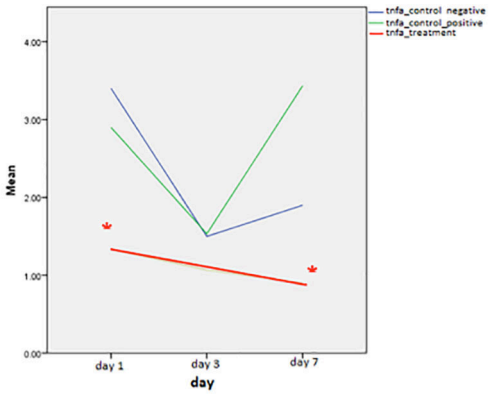

Figure 2. TNF- α cytokine level count comparison graphic between the studied groups.

Table 3 and Figure 3 show a significant decrease in IL-10 cytokines level on day-1 (p= 0.012), increasing significantly on the seventh day (p= 0.002).

Table 3

IL-10 Cytokine Level Count Analysis of The Studied Group

Groups Mean ± SD	First day	Third day Mean ± SD	Seventh-day Mean ± SD
Negative control	3.80 ± 1.81 [a]	3.33 ± 0.85	1.93 ± 0.88 [a]
Positive control	3.40 ± 1.02 [a]	3.67 ± 1.38	2.13 ± 1.06 [a]
Treatment group	1.43 ± 0.71 [b]	2.43 ± 0.75	4.47 ± 1.36 [b]
P value	0.012*	0.135	0.002*

*Statistically significant difference between a, and b
SD = Standard Deviation

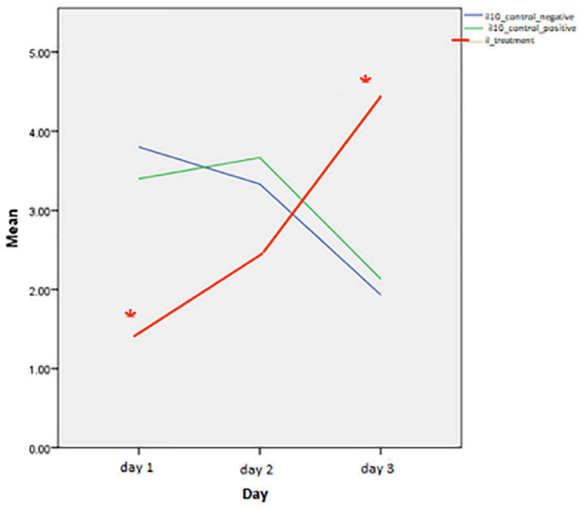

Figure 3. IL-10 cytokine level count comparison graphic between the studied groups.

DISCUSSION

Traditional Karo Oil regulates the inflammatory phase by accelerating the polarization of type-1 macrophages, marked by a decrease in the production of TNF-α pro-inflammatory cytokines significantly, into type-2 macrophages marked by an increase in the expression of IL-10 anti-inflammatory cytokines. The active ingredients of Karo Traditional Oils have been shown to regulate the inflammatory phase by suppressing the production of inflammatory cytokines characterized by low expression of pro-inflammatory cytokines TNF-α and IL-10 on the 1st post-treatment day 12, and stimulating the differentiation of monocyte cells and production of macrophage type-2 inflammatory cells and their activation, which is characterized by an increase in the anti-inflammatory cytokine IL-10 starting from the 1st to the 7th day after treatment while simultaneously releasing growth

factors which are the key regulation of the wound healing process (16,17). The α-pinene compound is a potent inflammatory regulatory compound, which is the main compound of Karo Traditional Oils regulating the inflammatory phase through inhibition of type-1 macrophages that express Cluster of Differentiation 86 (CD86) and pro-inflammatory cytokines such as TNF-α, IL-1, and IL-6 (16-18).

Inflammatory cells, especially those macrophages, play an essential role in the tissue repair process. Reduction in macrophage infiltration is closely related to the incidence of the prolonged wound healing phase (19). A significant increase in the number of inflammatory cells was found on the 1st post-treatment day in wounds treated with traditional Karo Oil compared to wounds treated with traditional Karo Oil solvent oil and wounds treated with PZ gauze, as shown in Figure.1. Typically, in wounds treated with PZ gauze, the inflammatory cells that come out first are neutrophil cells, and this will last for the first 72 hours after the wound occurs. The presence of neutrophil cells that extend in the wound will prolong the inflammatory phase because neutrophils release enzymes that degrade tissue so that the extracellular matrix deposit process and collagen synthesis to fill the wound tissue will be inhibited because the enzymes from these neutrophils degrade collagen (20). Meanwhile, this does not occur in wounds treated with traditional Karo Oil because the active ingredient content of this oil accelerating the change of neutrophils to macrophages in the first 24 hours and stimulates the differentiation of monocyte cells into inflammatory macrophage cells consistently from day 1 to day 7, which under normal circumstances began to occur on the 3rd day. This proves that the active compound in Karo Traditional Oils in the form of monoterpene compounds is a regulator of the inflammatory phase and also functions as a macrophage-activating substance (16,20).

The production of pro-inflammatory cytokines, in this case, represented by the low expression of TNF-α cytokines by type-1 macrophages from day 1 post-treatment to day 7 post-treatment showed a decreasing trend, whereas in the other two groups treated by solvent oil Karo Traditional Oils and moist gauze showed a decrease in pro-inflammatory cytokine TNF-α on day 1 to day 3 post-treatment and tended to increase again on day 7 post-treatment, as shown in Figure 2. α-pinene compounds and other terpene compounds from Karo Traditional Oils block the TLR 4 receptors on Iκβ kinase so that they inhibit Iκβ phosphorylation, this inhibits the activation of NFκβ, IKK-β, and MAPK suppression occurs through decreased MAPK phosphorylation (ERK and JNK) (21) so that the expression of pro-inflammatory mediators such as TNF-α is suppressed so as not to excess (22-24). TNF-α in this low level also plays a role in the re-epithelialization process by maintaining the stability of keratinocyte activation signals by cytokines IL-1 and stimulating K6 expression through transcription factors NFκβ and C/EBPβ and indirectly stimulating the secretion of cytokines FGF-7 by cells.

In this case, the content of bicyclic terpenes in traditional Karo oil, α-pinene, δ-3-carene, sabinene, and camphene has been shown to accelerate the resolution of inflammatory reactions through increased production of type-2 macrophages and anti-inflammatory compounds characterized by an increasing trend in expression. Cytokine IL-10 was significantly compared to the other two groups, from day-1 post-treatment to its peak at day 7 post-treatment, as shown in Figure 3 (17,22). The IL-10 cytokine is an anti-inflammatory cytokine to regulate the inflammatory phase, is anti-angiogenic, and a regulator of extracellular matrix deposition, which results in scar-free healing (25). With the discovery of a significant increase in the cytokine IL-10 on the 7th day, it is possible that wound healing in wounds treated by traditional Karo oil will be better aesthetically because it causes fine scars. Terpene compounds from traditional Karo oil stimulate TLR 3 receptors and inhibit the COX-2 pathway to stimulate the release of anti-inflammatory cytokines, this stimulates the polarization of macrophages from type-1 to type-2, and an anti-inflammatory response and healing process occurs (22). Therefore, it can be concluded that Karo Traditional Oils accelerates the polarization process of macrophage inflammation cells from Macrophages type-1 that express CD86 and pro-inflammatory cytokines such as TNF-α, IL-1, IL-6, and INF-γ, into type-2b macrophages, also known as type-2 macrophages or wound healing macrophages

that express CD 86 and the anti-inflammatory cytokine IL-10 with a significantly increased trend, growth factor TGF-β, and some MMP, and express the pro-inflammatory cytokine TNF-α, and IL-6 in small amounts.

The presence of type 2b macrophages expressing IL-10 and TGF-β will stimulate type 2c macrophages that express CD 206 and anti-inflammatory cytokines IL-10, MMP-9 IL-1β, and TGF-β in large amounts, and pro cytokines. -inflammatory IL-12 in small amounts, thus accelerating the transition of the wound healing process from the inflammatory phase to the proliferative phase where the wound treated with PZ gauze occurred after the 3rd day, but the wound treated with traditional Karo oil began to appear on the 3rd day (17,22,26).

CONCLUSION

Traditional Karo Oil regulates the inflammatory phase by accelerating the polarization of type-1 macrophages, marked by a decrease in the production of TNF-α pro-inflammatory cytokines significantly, into type-2 macrophages marked by an increase in the expression of IL-10 anti-inflammatory cytokines. As a result, it has been found that the re-epithelialization process in wounds smeared with Traditional Karo Oil since the third day after treatment and increased significantly on the 7th-day post-treatment.

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Conflicts of Interest

The authors declare no conflict of interest in this Traditional Karo Oil research.

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