



Topical administration of *Curcuma longa* L. extract gel increases M2 macrophage polarization and collagen density in skin excision

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ABSTRACT

While M1 macrophages initiate wound healing by inducing the inflammation phase, M2 macrophages are crucial in the proliferation phase by producing growth factors that accelerate new tissue formation. Turmeric (*Curcuma longa* L.) is known to accelerate wound closure; however, mechanisms underlying the wound healing properties of *C. longa* L. remain to be documented. Therefore, this study investigated the mechanisms by which *C. longa* L. accelerates wound healing. Here, an experimental study was conducted using 30 male Swiss Webster mice. *Curcuma longa* L. extract was applied on the wound bed tissue starting on day 3 after wound induction. The *Arginase-1* (*Arg-1*) and inducible nitric oxide synthase (*iNOS*) (M1 and M2 markers) gene expressions from wound skin tissue were analyzed, while collagenesis during tissue repair was observed by histopathology analysis of excised skin on day 6 after wound induction. Upregulation of *Arg-1* in the treated group was observed on day 6 after wound induction compared to the positive control ($p = 0.0394$) and the negative control groups ($p = 0.0313$). In addition, the *Arg-1/iNOS* ratio revealed a significant increase of M2 polarization in the *C. longa* L.-treated wound compared to negative controls ($p < 0.0001$), but not significant when compared with positive controls ($p = 0.0535$). Similarly, collagen density was significantly higher in the *C. longa* L.-treated wound than the negative control ($p = 0.0418$) and the positive control ($p < 0.0001$). These results suggest that administration of *C. longa* L. extract gel improved wound tissue repair on skin excision by inducing M2 polarization on wound tissue during healing.

INTRODUCTION

Acute and chronic wounds do not only cause pain but also significantly impose an economic burden (Guest *et al.*, 2017; Järbrink *et al.*, 2017). As a result, productivity, business returns, and tax revenue are greatly affected (Nussbaum *et al.*, 2018; Sen *et al.*, 2009). Given that the rate of accidents is very high in Indonesia, research into wound care has become a great priority (Jusuf *et al.*, 2017; Karina *et al.*, 2019; Tarawan *et al.*, 2017).

Tissue repair in wound healing consists of three consecutive phases including inflammatory, proliferation, and maturation phases. Macrophages play an important role in all the phases of tissue repair (Shedoeva *et al.*, 2019). Following activation, macrophages are polarized into two types, i.e., classically activated macrophages (M1) and alternatively activated macrophages (M2) (Yuliani *et al.*, 2018). Inhibition of macrophage activity has been reported to be associated with prolonged wound healing time (Shedoeva *et al.*, 2019). Similarly, as described among diabetic patients, persistent M1 macrophages in wound tissues and inhibition of M2 macrophage polarization in wound tissues make it difficult for chronic wounds to heal (Shah *et al.*, 2017). Prolonged wound healing results in prolonged treatment and eventually increases the treatment cost. In view of

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this, a new breakthrough in wound care is required to produce effective, efficient, and inexpensive treatment options.

Medicinal plants for wound healing are not only cheap and affordable but also safe to use. These plants induce healing and regeneration of lost tissues by various mechanisms (Gao *et al.*, 2015). According to the 2014 Horticultural Data, *Curcuma longa*, commonly known as turmeric rhizome, is the second highest biopharmaceutical plant in Indonesia (Jacob *et al.*, 2007).

Furthermore, turmeric rhizome plants are widely used as an additive to food and also as a medicine for treating tissue inflammation problems (Chen *et al.*, 2015). The turmeric rhizome contains curcumin, which modulates inflammatory responses through downregulation of cyclooxygenase-2 activity, lipoxygenase, and inducible nitric oxide synthase (*iNOS*) enzymes (Riquelme *et al.*, 2013). In addition, in a macrophage cell line (Raw264.7), curcumin has been shown to induce polarization of M2 macrophages (Krzyszczczyk *et al.*, 2018).

Since curcumin in turmeric rhizome has been identified to modulate the inflammatory response and promote the polarization of M2 macrophages *in vitro*, investigating the effect of topical administration of turmeric rhizome extract gel on wound healing is warranted.

MATERIALS AND METHODS

Animal model

Thirty (30) Swiss Webster male mice, each weighing 22–26 g and about 8–10 weeks old, were obtained from the Faculty of Biology, Bandung Institute of Technology. Previously, these mice had been adapted for 1 week. The mice were divided into three groups and each group consisted of 10 mice. For wound induction, two circular skin excisions were performed on the back of each mouse using a biopsy punch with a diameter of 6 mm. After excision, the wound was cleaned with physiological NaCl, dried with sterile gauze, and covered with a transparent dressing; then, they were covered with dry gauze and plastered. Treatment of the wound was carried out on the 3rd day after the injury. In the positive control group, the open wounds were treated with tulle (polymyxin B sulfate + neomycin sulfate + bacitracin zinc). In the negative control group, the open wounds were given blank gel. In the last group, the open wounds of the mice were given turmeric (*C. longa*) rhizome extract gel. All mice were killed by cervical dislocation technique on the 6th day after the injury for excision and collection of the wounded skin tissue. For histopathology observation, the skin tissues collected were fixed for microscopic observation. One part of the skin tissue was taken and stored in liquid nitrogen immediately for *Arginase-1* (*Arg-1*) and *iNOS* (M2 markers) gene expressions analysis using quantitative Polymerase Chain Reaction (qPCR).

Turmeric rhizome extract gel

Turmeric rhizome (*C. longa*)-based extract gel (3%) was provided by the Pharmacy Laboratory, Faculty of Pharmacy, Universitas Padjadjaran.

Histopathology (Collagen) observation

Collagenesis was observed by histopathology analysis of excised skin on day 6 after wound induction. Collagen was

observed through Mason's trichrome staining as zones of purple strands. In this study, collagen density was measured by taking an average of three collagen fibers that appeared intact on the two edges and center of the wound using a micrometer under a light microscope with a magnification of 10x and 40x objective lens. Scoring 0–4 indicates more solid collagen.

RNA extraction

The collected skin tissues were transferred into a homogenizer tube containing ceramic beads and 1 ml of Qiazol was added (Qiagen, Germany). Tissue lysis was carried out using Precellys homogenizer (6,000 rpm). The lysate was then transferred into 1.5 ml of microcentrifuge tube and 100 μ l of chloroform was added gently. The tube was shaken for 10 seconds and incubated for 10 minutes at room temperature. The centrifugation was then carried out at 12,000 \times g for 12 minutes at 4°C. After three phases were formed, 350 μ l of the clear top layer consisting of the ribonucleic acid (RNA) was transferred into new tubes and washed with one volume of 70% ethanol. Afterward, RNA precipitate was transferred into a minicolumn tube, which consisted of RB column and 2 ml collection tube (Geneaid RNA Extraction kit, Taiwan) and centrifuged for 1 minutes with 14,000 \times g at 4°C. The next step was performed according to the manufacturer's (Geneaid, Taiwan) instructions. The extracted RNA was quantified using a spectrophotometer and the concentration of RNA was measured using absorbance at 260 and 280 nm.

Gene expression analysis

The *Arg-1* and *iNOS* (M1 and M2 markers) gene expressions from wound skin tissues were analyzed using the quantitative real-time PCR method. At least 4.2 μ g/ml from the total RNA was reverse-transcribed to be complementary deoxyribonucleic acid (cDNA) using ReverTra Ace® qPCR RT Master Mix (Toyobo, Japan), according to the manufacturer's instruction. PCR was then carried out using Thunderbird® SYBR® qPCR Mix (Toyobo, Japan) kit, according to the manufacturer's instruction. Real-time PCR was performed using Rotorgene Q cyclor (Qiagen, Germany). The gene expressions assessed were *Arginase-1* (*Arg-1*) as a marker for M2 macrophage and *iNOS* as M1's marker. The gene expressions were analyzed using the following formula: $2^{-\Delta\Delta Ct}$, in which $\Delta\Delta Ct = (Ct \text{ gene of interest} - Ct \text{ reference gene}) \text{ treatment group} - (Ct \text{ gene of interest} - Ct \text{ reference gene}) \text{ control group}$.

The degree of gene expression was normalized with housekeeping gene β -actin as a reference gene and the result presented as a fold change to the control group. The primer sequences (mouse) are presented in Table 1. Quantitative real-time PCR was carried out at 95°C for 10 minutes, followed by at least 40 cycles at 95°C for 20 seconds and 60°C for 1 minutes.

Ethical statement

This study was approved by the Research Ethics Committee, Universitas Padjadjaran (705/UN6.C.10/PN/2017). All efforts were made to minimize the suffering of animals during the experimental procedures.

Statistical analysis

The Shapiro–Wilk test was used for the normality analysis and the Levene test was used for the homogeneity

analysis. Analysis of the mean difference between the two groups based on the results of this normality test used independent *t*-test for variables with normal distribution or the analysis of variance (ANOVA) test, followed by Tukey's multiple comparison test to compare more than two groups. Meanwhile, for variables with abnormal distribution, the Mann–Whitney U test was used for comparison between two groups. The analysis was performed with statistical software GraphPad Prism 8.

RESULTS

Histopathology

Representative pictures of microscopic skin tissue among positive control, negative control, and *C. longa* treatment

groups are shown in Figure 1. The expression of collagen was highest in the *C. longa* treatment group compared to the positive ($p < 0.0001$) and negative control ($p = 0.0418$) groups. However, there was no statistical difference between the positive and negative control groups (Fig. 2).

Gene expression marker analysis for M1 and M2

Gene expression analysis on *Arg-1* showed a significant increase in the *C. longa* treatment compared to the positive control ($p = 0.0394$) and negative control ($p = 0.0313$) (Fig. 3). Statistical analysis of the *iNOS* gene expression showed a decreasing trend in the *C. longa* treatment compared to the positive control and the negative control, but it was not significant (Fig. 4).

Table 1. Primer sequences used for quantitative real-time PCR.

Gene	Forward (5'–3')	Reverse (5'–3')
Mouse <i>Arginase-1</i>	CCTATGTGTCATTTGGGTGGA	CAGGAGAAAGGACACAGGTTG
Mouse <i>iNOS</i>	CCAAGCCCTCACCTACTTCC	CTCTGAGGGCTGACACAAGG
Mouse β -actin	AGAGGGAAATCGTGCGTGAC	CAATAGTGATGACCTGGCGGT

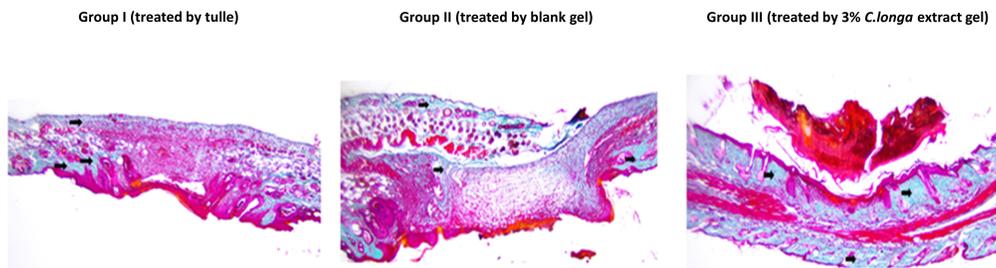


Figure 1. Microscopic appearance of skin on the 6th day after injury; group I was positive control, group II was negative control, and group III was treated by 3% *C. longa* extract gel. The histopathological observation shows more collagen in the tissues that were treated with 3% *C. longa* extract gel.

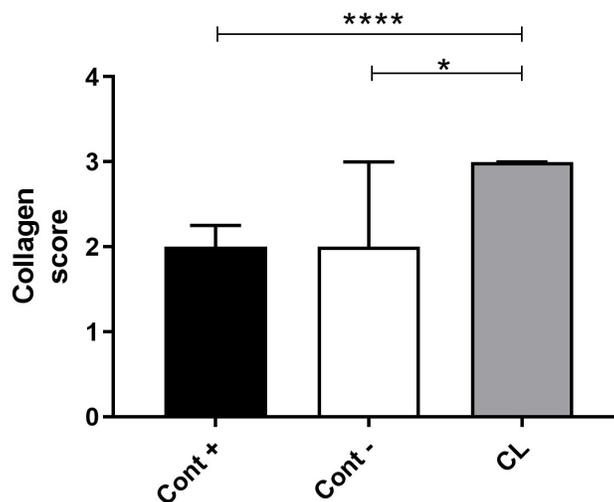


Figure 2. Comparison graph of collagen density in positive controls (Cont +), negative controls (Cont -), and *C. longa* (CL). Data are presented as median \pm interquartile range. Statistical analysis between two groups was tested using Mann–Whitney U test; $*p < 0.05$; $****p < 0.0001$.

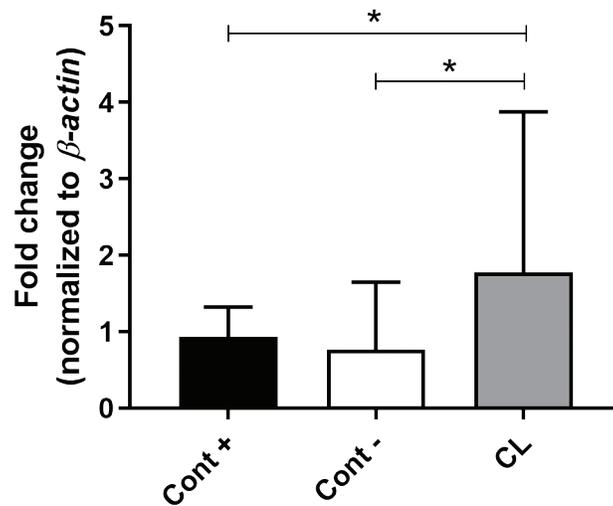


Figure 3. Comparisons of *Arginase-1* gene expression in positive controls (Cont +), negative controls (Cont -), and *C. longa* (CL). Graph is presented as median with interquartile range. Statistical analysis between two groups was tested using Mann–Whitney U test; * $p < 0.05$.

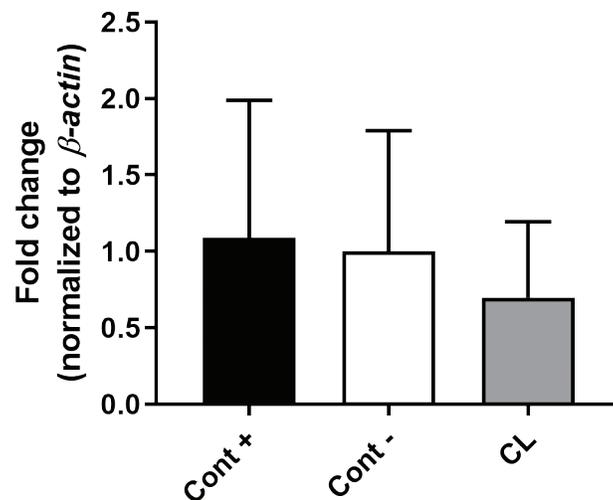


Figure 4. Comparison of *iNOS* gene expression in positive controls (Cont +), negative controls (Cont -), and *C. longa* (CL). Graph is presented as mean with SD. Statistical analyses were tested using ANOVA, followed by Tukey's multiple comparison test.

Comparison of *Arg-1* and *iNOS* gene expression ratios (Fig. 5) showed a significantly higher ratio in *C. longa* compared to negative controls ($p < 0.0001$), but it was not significant when compared with positive controls ($p = 0.0535$).

DISCUSSION

In this study, we observed that *C. longa* has the ability to increase wound healing properties through the upregulation of *Arg-1* gene in M2 polarized macrophages. The highest collagen fibers were formed in tissues that were treated with *C. longa* compared with the other groups. The higher amount of collagen fibers in mice of the *C. longa*-treated group could be due to the presence of beta-diketone and polyphenolic functional groups which act as

strong antioxidants (Sha *et al.*, 2019; Shedoeva *et al.*, 2019). As a result, lipid peroxidation decreases but vascularity and collagen synthesis, as well as cross-linking of collagen significantly improve (Yuliani *et al.*, 2018). *Curcuma longa* has anti-inflammatory properties and, therefore, topical application of *C. longa* gel extracts may have negative effects on the inflammatory phase in wound healing (Shah *et al.*, 2017; Xue *et al.*, 2018). To avoid this occurrence, the gel extract of *C. longa* was applied to the wounds of the mice on the 3rd day after injury. This study showed that *Arg-1* significantly increased in the mice treated with *C. longa* compared to the positive controls and negative controls, and therefore, coupled with increased collagen density, wound healing was accelerated in the *C. longa*-treated group. These findings

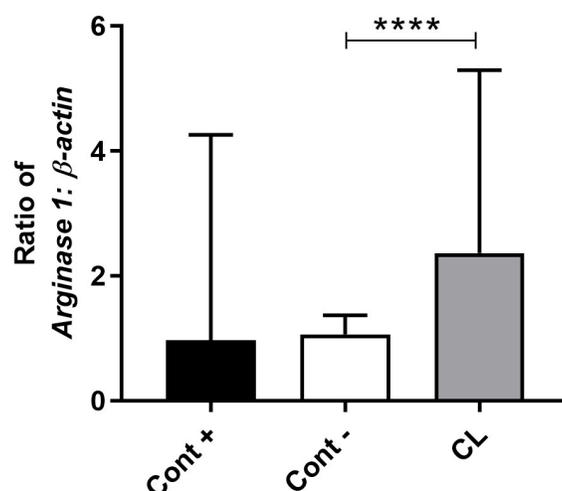


Figure 5. Comparison of the ratio of *Arginase-1* gene expression with *iNOS* in positive controls (Cont +), negative controls (Cont -), and *C. longa* groups. Graph is presented as median with interquartile range. Statistical analysis between two groups was tested using Mann–Whitney U test; **** $p < 0.0001$.

corroborate with several studies, wherein curcumin increased IL-4 and IL-13 proteins and messenger RNA (mRNA) expression which induced M2 polarization by upregulation of M2 markers (*Arg-1*, macrophage mannose receptor (MMR) gene, and Peroxisome proliferator-activated receptor gamma (PPAR- γ)) in Raw264.7 macrophages treated with *C. longa* (Gao *et al.*, 2015; Jacob *et al.*, 2007). According to other report, the inhibition of arginase activity significantly delayed wound healing time, and therefore, *C. longa* gel extracts can accelerate wound closure (Campbell *et al.*, 2013). Generally, *iNOS* is induced by M1 macrophages and its expression is often low in human blood monocyte-derived macrophages (Ley, 2017; Riquelme *et al.*, 2013). In this study, *iNOS* expression decreased in mice treated with *C. longa* compared with the negative controls and the positive controls. This may suggest that the local inflammatory processes and the resultant oxidative stress during injury may be reduced by topical application of *C. longa* gel extracts (Graf *et al.*, 2017), thereby improving and hastening the healing of wounds (Choudhary *et al.*, 2018). This finding concurs with studies conducted by Ibrahim *et al.* (2018) and Mohanty *et al.* (2017), where *C. longa* gel extracts decreased the expression of *iNOS* and improved tissue repair, as well as the healing process.

Even though *iNOS* protein expression can hamper wound healing, it is a very essential enzyme that produces nitric oxide, a proinflammatory molecule that inactivates and destroys infectious microbes and as a result protects humans from diverse infectious diseases (Bogdan *et al.*, 2000; Ellis *et al.*, 2018). Since *iNOS* also helps the host, it is imperative to treat wounds with medications that balance the *Arg-1* and *iNOS* ratio that will ensure that the benefits of both proteins are maintained but with decreased toxicity of *iNOS*. In this study, we revealed that the ratio of *Arg-1* to *iNOS* was significantly higher in the *C. longa* treatment group than the negative controls; nevertheless, there was no substantial difference between *C. longa* treatment group and the positive controls. A high ratio of *Arg-1* to *iNOS* activity improves the healing of wounds (Campbell *et al.*, 2013), and by the findings

of our study, it can be suggested that *C. longa* gel extracts will promote tissue repair and wound healing.

CONCLUSION

Based on this study, topical application of the turmeric rhizome (*C. longa*) gel extract accelerates the proliferation phase in the acute wound healing process through the induction of M2 macrophages and increasing collagen density in the wound tissue. However, further research is needed to explore more about the benefits of turmeric rhizome (*C. longa*) gel extract on the maturation/remodeling phase of the wound healing process.

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CONFLICT OF INTEREST

The authors declare no conflict of interests.

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REFERENCES

- Bogdan C, Röllinghoff M, Diefenbach A. The role of nitric oxide in innate immunity: no in innate immunity. *Immunol Rev*, 2000; 173(1):17–26.
- Campbell L, Saville CR, Murray PJ, Cruickshank SM, Hardman MJ. Local arginase 1 activity is required for cutaneous wound healing. *J Invest Dermatol*, 2013; 133(10):2461–70.
- Chen H, Shi R, Luo B, Yang X, Qiu L, Xiong J, Jiang M, Liu Y, Zhang Z, Wu Y. Macrophage peroxisome proliferator-activated receptor γ deficiency delays skin wound healing through impairing apoptotic cell clearance in mice. *Cell Death Dis*, 2015; 6(1):e1597.

- Choudhary V, Shivakumar HG. A review on curcumin: wound healing properties and biomarkers of wound healing. *Int Res J Pharm*, 2018; 9(9):1–5.
- Ellis S, Lin EJ, Tartar D. Immunology of wound healing. *Curr Dermatol Rep*, 2018; 7(4):350–8.
- Gao S, Zhou J, Liu N, Wang L, Gao Q, Wu Y, Zhao Q, Liu P, Wang S, Liu Y, Guo N, Shen Y, Wu Y, Yuan Z. Curcumin induces M2 macrophage polarization by secretion IL-4 and/or IL-13. *J Mol Cell Cardiol*. 2015; 85:131–9.
- Graf BL, Jaja-Chimedza AL, Raskin IL. Comparative anti-inflammatory effect of moringa-derived isothiocyanate versus curcumin *in vitro* and *in vivo*. *FASEB J*, 2017; 31(S1):972.22.
- Guest JF, Vowden K, Vowden P. The health economic burden that acute and chronic wounds impose on an average clinical commissioning group/health board in the UK. *J Wound Care*, 2017; 26(6):292–303.
- Ibrahim NI, Wong SK, Mohamed IN, Mohamed N, Chin KY, Ima-Nirwana S, Shuid AN. Wound healing properties of selected natural products. *Int J Environ Res Public Health*, 2018; 15(11):1–23.
- Jacob A, Wu R, Zhou M, Wang P. Mechanism of the anti-inflammatory effect of curcumin: PPAR- γ activation. *PPAR Res*, 2007; 2007:1–5.
- Järbrink K, Ni G, Sönnergren H, Schmidtchen A, Pang C, Bajpai R, Car J. The humanistic and economic burden of chronic wounds: a protocol for a systematic review. *Syst Rev*, 2017; 6(1):15.
- Jusuf A, Nurprasetio IP, Prihutama A. Macro data analysis of traffic accidents in Indonesia. *ITB J Public*, 2017; 49(1):132–43.
- Karina, Samudra MF, Rosadi I, Afini I, Widyastuti T, Sobariah S, Remelia M, Puspitasari RL, Roslana I, Tunggadewi TI. Combination of the stromal vascular fraction and platelet-rich plasma accelerates the wound healing process: pre-clinical study in a Sprague-Dawley rat model. *Stem Cell Investig*, 2019; 6:18.
- Krzyszczak P, Schloss R, Palmer A, Berthiaume F. The role of macrophages in acute and chronic wound healing and interventions to promote pro-wound healing phenotypes. *Front Physiol*, 2018; 9:1–22.
- Ley K. M1 means Kill; M2 means heal. *J Immunol*, 2017; 199(7):2191–3.
- Mohanty C, Sahoo SK. Curcumin and its topical formulations for wound healing applications. *Drug Discov Today*, 2017; 22(10):1582–92.
- Nussbaum SR, Carter MJ, Fife CE, DaVanzo J, Haight R, Nussbaum M, Cartwright D. An economic evaluation of the impact, cost, and medicare policy implications of chronic nonhealing wounds. *Value Health*, 2018; 21(1):27–32.
- Riquelme P, Tomiuk S, Kammler A, Fändrich F, Schlitt HJ, Geissler EK, Hutchinson JA. IFN- γ -induced iNOS expression in mouse regulatory macrophages prolongs allograft survival in fully immunocompetent recipients. *Mol Ther*, 2013; 21(2):409–22.
- Sen CK, Gordillo GM, Roy S, Kirsner R, Lambert L, Hunt TK, Gottrup F, Gurtner GC, Longaker MT. Human skin wounds: a major and snowballing threat to public health and the economy. *Wound Repair Regen*, 2009; 17(6):763–71.
- Sha AM, Garib BT. Antibacterial effect of curcumin against clinically isolated porphyromonas gingivalis and connective tissue reactions to curcumin gel in the subcutaneous tissue of rats. *BioMed Res Int*, 2019; 2019:1–14.
- Shah A, Amini-Nik S. The role of phytochemicals in the inflammatory phase of wound healing. *Int J Mol Sci*, 2017; 18(5):1068.
- Shedoeva A, Leavesley D, Upton Z, Fan C. Wound healing and the use of medicinal plants. *Evid Based Complement Alternat Med*, 2019; 2019: 1–30.
- Tarawan VM, Mantilidewi KI, Dhini IM, Radhiyanti PT, Sutedja E. Coconut shell liquid smoke promotes burn wound healing. *J Evid Based Complement Altern Med*, 2017; 22(3):436–40.
- Xue Q, Yan Y, Zhang R, Xiong H. Regulation of iNOS on immune cells and its role in diseases. *Int J Mol Sci*, 2018; 19(12):1–13.
- Yuliani S, Mustofa, Partadiredja G. Turmeric (*Curcuma longa* L.) extract may prevent the deterioration of spatial memory and the deficit of estimated total number of hippocampal pyramidal cells of trimethyltin-exposed rats. *Drug Chem Toxicol*, 2018; 41(1):62–71.

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