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# The Effects of Noni (*Morinda Citrifolia*) Ethanol Extract Cream on Collagen Deposition in Incisional Wound Healing of Male Wistar Rats

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**Abstract:** ***Incisional wounds, such as post-surgery wounds are traumas that could reduce quality of life of patients and cause many complications. While often disturbed by many factors, reactive oxygen species (ROS) and unending inflammation stage is two of many contributing factors that can delay wound healing. Therefore, new natural phytochemical source is needed to accelerate the healing of wounds and minimize the risk of complications. On this experimental analytic research with control group post-test research design, twenty-four male wistar rats divided into four groups. The first group is P1 (Incisional Wound + Placebo Cream) as control, P2 (Incisional Wound + 5% Noni extract cream), P3 (Incisional Wound + 10% Noni extract cream), P4 (Incisional Wound + 20% Noni extract cream). At the end of fifteenth day of wound healing, all samples are terminated and the scar tissue is observed under the microscope with Picrosirius Red (PSR) staining. Groups that are treated with concentrations of 5%, 10%, and 20% Noni extract cream have higher mean percentage of collagen deposition compared with the placebo group. Mean percentage of group P1 is 42,49%, P2 is 54,18%, P3 is 64,89%, and P4 as high as 72,22%. Through this study, topical application of noni ethanol extract cream on incisional wounds of wistar rats gained an evidence on accelerating healing and increasing collagen deposition of the wounds compared to base cream applications only.***

**Keywords:** **N**oni, quercetin, incisional wound, collagen.

## 1. Introduction

Skin as the main part of the integumentary system, is the biggest and heaviest organ that composes around 15% of total human body weight. It functions primarily as the protective lining of almost every part of the body, alongside with many important roles such as excretion and regulating body temperature [1]. Being the outermost part of the body, skin is prone to many unfavorable conditions. Pathogenic infections, trauma, pollution induced damage, accelerated aging, and malignancy are the impacts of both external and internal factors that could harm the skin [2]–[6]. Incisional wound is a clean cut wound type that is common and can be caused either by everyday sharp objects such as broken glass utensils, knifes, and scissors, or by medical procedures such as surgery [7]. This type of wound relatively heals faster and leaves less extensive scar tissue than more irregular shaped wounds, but when the healing process is disturbed, by factors such as oxidative stress and prolonged inflammation, complications such as secondary infections may occur [8], [9].

Inflammatory cells and chemical mediators begin to act as soon as the wound is formed. Through four stages of wound healing, proinflammatory cells such as platelets, neutrophils, macrophages, and lymphocytes come to congregate at the wound site. Other than cleaning up debris and pathogens from the disruption of the skin tissue, these cells also produce growth factors that take important roles in wound healing such as VEGF, PDGF, FGF and TGF-β [10].

Transforming growth factor beta 1 or TGF-β1 is a dimer glycoprotein cytokine that presents as the most abundant of three isomers of TGF-β group. This cytokine is produced by macrophages, fibroblasts, platelets, and some other immune cells in a feedback loop manner [11]. In wound healing process, TGF-β1 level rises when epithelialization starts on the proliferation stage. It is also known that in an *in vitro* study, TGF-β1 could put cell cycle into a total stop at G1 phase. From these theories, it can strongly be presumed that TGF-β1 acts both as a stimulator and inhibitor of wound healing, depends on the phase and needs of local tissues [11], [12]. Deficiency of this growth factor can lead to disturbance of wound healing, as it has been reported that TGF-β1 induces angiogenesis, production, and maturation of collagen in extracellular matrix repair. TGF-β1 also stimulates *de novo* serine synthesis in collagen production through shorter pathway [13], [14].

Antioxidants are known to diminish high oxidative stress levels that can disrupt the normal physiology of the body. Studies involving pre-eclampsia and UV-B radiation exposed murine models has shown good results in restoring normal physiology of wistar rats [15], [16]. Therefore, application of antioxidant to accelerate and optimize wound healing is considered a rational treatment.

Noni (*Morinda citrifolia*) is a tropical plant that is abundantly grows all across the Polynesia and Southeast Asia. Its fruit, leaves, and roots are common to be consumed as food and traditional medicine in many countries. Noni fruit contains more than two hundred phytochemical substances including quercetin, a flavonoid derivative compound that is contained as much as 0,015 mg into 0,200 mg in one gram of fruit [17], [18]. As an antioxidant, quercetin has the ability to engulf free radicals in cells and

protect epithelial cells, fibroblasts, keratinocytes, and endothelial cells from oxidative stress [19]. Quercetin as a natural phytochemical also has the attribute to regulate TGF-β1 levels which can accelerate healing and weave collagen fiber with better arrangement on skin wounds [20].

Based on good evidences regarding active substances in noni fruit, efficacy of topical application of noni ethanol extract cream in incisional wound healing is determined by conducting an experimental research using murine models with incisional wounds.

## 2. Materials and Methods

### 2.1 Establishment of incisional wounds on animal models

Twenty-four male adult wistar rats (150-200 g) were used in this study. All rats were cared under controlled condition with temperature at 25 ± 2°C, relative humidity of 50 ± 15% relative humidity, and normal photoperiod (12-hours light-dark cycle). Standard mouse pellet and tap water were provided ad libitum for all wistar rats. On the first day, all rats were given an incisional wound on dorsal area. Ketamine 40 mg Xylazine with dose of 0,86 mL/kgBW were administered intramuscularly five minutes prior to incisional wound formation. After a portion of dorsal fur on lumbar area were shaved, the incisional wounds were made starting approximately on the first lumbar vertebrae, incised towards the caudal as long as 1,5 cm using a new Gilette® double-edge razor blades with estimate width of 0,1 cm.

### 2.2 Noni ethanol extract cream construction method

One kilogram of noni fruits that were harvested from a noni tree in Denpasar. The fruits were skinned and were immediately cut into small pieces, then chopped finely using a conventional blender. A hundred grams of sample was inserted into a macerator, added with 95% of ethanol with the ratio of 1:3, then left to sit for 24 hours. The extract then filtered, the dregs were soaked again and stirred in ethanol 95% with ratio of 1:2 for 2 hours then was left to sit for 24 hours, while the liquid were mixed with water, glycerin, and propylene glycol. Soaked dregs then evaporated on 70°C until thick extract is obtained. The two liquors then combined and stirred on 1000 rpm until finely mixed. The final extract then stored at -4°C, shortly mixed with base cream with three different concentrations of 5%, 10%, and 20% and stored on -4°C.

### 2.3 Experimental protocol

Twenty-four wistar that has been given incisional wounds were divided into four groups and cared separately by each group. The first group, P1, is a control group treated with placebo cream, P2 is treatment group with noni ethanol extract cream with concentration of 5%, P3 group is treated with 10% noni ethanol extract concentration in the cream, and P4 with 20% noni ethanol extract concentration in cream.

As soon as the wounds were made on first day, all rats were applied with their respective cream based on their intervention group twice a day at around 8.00am-9.00am and 2.00pm-3.00pm local time with the amount of 0,05 mg/cm$^2$ until the fifteenth day.

### 2.4 Collagen observation

On the fifteenth day after 28 times of topical application of each cream, all rats were euthanized and the dorsal skin containing wound area were excised at the size of 2×0,5 cm and immediately put into 10% buffered formaline. The skin samples then made into paraffin blocks and stained using Picrosirius Red (PSR) staining.

The first step of PSR staining is deparaffinization, the samples were put into xylene reagents for 2-3 minutes, then put into three ethanol solution for 2-3 minutes respectively, and washed three times using di$H_2O$, then patted dry using clean gauze. The deparaffinized samples then stained in Sirius red coloring for an hour, and subsequently washed twice with wash solution (ten dips or sit for 2-3 minutes each). On dehydration stage, the samples dipped in variant solutions in a sequential list; di$H_2O$, 70% alcohol solution, 90% alcohol solution, 95% alcohol solution, 100% alcohol solution (v/v) ethanol (ten dips or 2–3 min incubation each). Hundred percent ethanol solution is rinsed to the slide to ensure that all water is removed. Ethanol then replaced by xylene by dipping the slide into two xylene reagents, then the slide is mounted with organic mounting medium and coverslips [21].

Under the microscope, collagen deposition percentage of each sample was observed with Cx41 Olympus® microscope and photographed in JPEG format using Optilab PRO (Indonesia) camera on magnification of 20 times with six fields of view. Collagen fibers then counted with Adobe Photoshop CC 2015, where collagen fibers having bright red color and other tissues are yellow. Collagen deposition percentage (CDP) is then obtained by calculating the proportion of collagen fiber pixels in the image by its surrounding tissue pixels, with the formula as shown below.

$$\text{CDP} = \frac{Collagen\ fiber\ pixels}{Total\ tissue\ pixels} * 100\% \quad (1)$$

### 2.5 Analytical data procedure

All samples then analyzed using IBM Statistical Package for the Social Sciences (SPSS) Statistics 23.0 software to know test of normality result (using Shapiro-Wilk test, with ≤50 samples and normally distributed data with p≥0,05), Levene's test is used to test the normality of data, and One-way ANOVA is used to compare the data within and between groups, after the samples were confirmed to be homogenous and normally distributed. With outstanding significant differences, normal distribution, and homogeneity in the results, Post-Hoc Least Significance Difference (LSD) test was done as a complement.

## 3. Results

Six sample datas from each intervention group was calculated to determine the mean percentage of collagen deposition of each group, then analyzed using One-way ANOVA. The collagen deposition percentage shown to increase directly proportional with noni ethanol extract cream concentration.

**Table 1:** Mean percentage of collagen deposition in each intervention group

| Group | N | Collagen Deposition Percentage (%) | SD (%) | *F* | p |
|---|---|---|---|---|---|
| P1 | 6 | 42,49 | 2,822 | 159,804 | ,000 |
| P2 | 6 | 54,18 | 2,132 | | |
| P3 | 6 | 64,89 | 2,389 | | |
| P4 | 6 | 72,22 | 2,647 | | |

In picrosirius red staining shown in Figure 1, collagen fibers are stained red whereas other skin tissues such as hair follicles and glands are colored yellow. Collagen fiber is seen the thinnest in P1 group, rising in density together with the rise of noni extract concentration percentage, showing visibly that P4 has the densest collagen deposition.

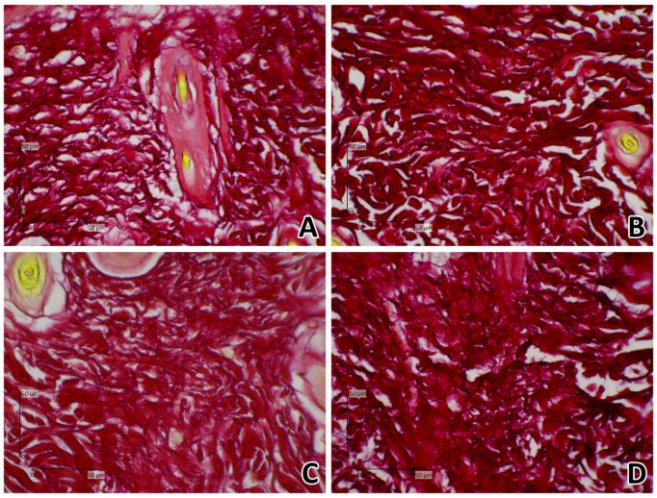


**Figure 1:** Representative photomicrograph of wound tissue with picrosirius red (PSR) staining (20×). (A) P1 group, (B) P2 group, (C) P3 group, (D) P4 group.

Post hoc analysis found that mean differences between two groups showed significant difference ($p<0,05$) among all statistical analysis. The highest mean difference between two adjacent intervals was found between placebo group and intervention group with the lowest concentration of noni in cream of 5% (11,69%) and the lowest mean difference was found between noni ethanol extract concentration of 10% and 20% (7,33%). Almost all neighboring group collagen density percentage showed mean difference more than 10%, as observed from P1 & P2 group and P2 & P3 group. Despite showing less mean difference increment from P3 group (7,3%), P4 group still showed significant difference ($p<0,05$) while analyzed with previous intervention group.

**Table 2:** Mean differences of collagen deposition percentage between two groups.

| Groups | Mean Difference (%) | p | Interpretation |
|---|---|---|---|
| P1 and P2 | 11,68833 | 0,001 | Significant |
| P1 and P3 | 22,39667 | 0,001 | Significant |
| P1 and P4 | 29,72167 | 0,001 | Significant |
| P2 and P3 | 10,70833 | 0,001 | Significant |
| P2 and P4 | 18,03333 | 0,001 | Significant |
| P3 and P4 | 7,32500 | 0,001 | Significant |

Serial wound picture was also taken as complementary data and displayed on Figure 2 to signify the healing process modification on a specific rat from each group for every three days starting from day three until pre-termination.

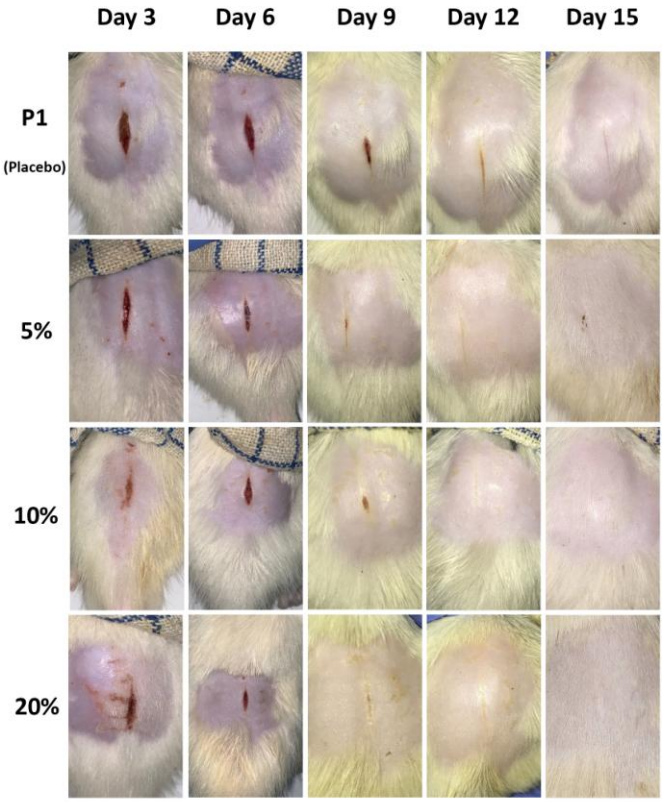


**Figure 2:** Differences of healing process of each group indicated by macroscopic wound closure of one specific rat from each group.

In groups with noni ethanol extract cream intervention, macroscopic healing of incisional wounds showed a satisfying result, particularly on the ninth day where rat from base cream-treated groups still had blood relatively wide blood post-wound blood clot formation compared to the other groups. Furthermore, on the fifteenth day after 28-times of topical administration of each respective cream, the placebo group still had a small slit compared to the others which had excellent healings.

## 4. Discussion

The evidences of this study have shown that active substances in noni fruit extract has an effect in accelerating and regulating extracellular matrix cell deposition. Other than quercetin as the main antioxidant, other some vitamins contained in noni fruit that are also potential antioxidants

such as β-carotene, vitamin C, and vitamin E may also helped to regulate wound healing in this experiment [22]. Several important phytochemical agents like phenol, scopoletin, rutin, and deacetylasperulosidic acid also have strong possibility of taking role [17].

Along with increment of noni ethanol extract composition in topical creams that were applied, collagen deposition percentage in wound tissues rises harmoniously. This condition can occur because of antioxidant activity of quercetin and other nutrients that could suppress healing process hampering oxidative stress level. Moreover, TGF-β1 regulating attribute of quercetin also plays important role in balancing post-trauma inflammation period and intensity [11], [12]. As a cytokine that maintains normal physiology of the cells, TGF-β1 could also support cell proliferation and collagen deposition [23], [24]. Being a group of flavonoid antioxidants, quercetin also have another mechanism of action by giving hydrogen ions so that the toxicity of free radicals can be neutralized [25]. In addition of being an antiinflamatory agent, quercetin also have the ability to work by inhibiting COX-2 arachidonic acid pathway, COX-1 pathway, and 12-LOX pathway from arachidonic acid metabolism. Other inflammatory mediators such as 12-HHT, TXB2, and 12-HETE are also decreased by synthesis inhibition [26].

Previous studies also revealed good results of quercetin usage on wound healing. Cahaya *et al.* proved that topical application of quercetin isolate gel in treating IIA degree burns of wistar rats has similar efficacy with neomycin sulfate gel application, thus could be an alternative to minimize antibiotic usage and prevent antimicrobial resistance [27]. Aboud *et al.* attempted to combine oral quercetin with low level laser therapy (LLLT) on albino rats with diabetic wounds and managed to bring growth factor rises for longer time than LLLT alone, therefore closes the wound faster and increases collagen deposition in wound [28].

Several researches also studied about noni fruit effects in wound healing. Senger and Cao explained about the action mechanism of noni fruit juice in helping diabetic wound healing. Noni fruit juice contains 4-ethyl catechol, 4-vinyl catechol, and 4-methyl catechol that functions to activate Nrf-2 gene which stimulate the healing of diabetic wound [29]. Yilmazer *et al*. reveals that flavonoids and coumarin in noni fruit juice has anti-inflammatory and antioxidant traits that are strong enough to cure paw edema on female wistar rats via nitric oxide pathway and prostaglandin E2 pathway [30]. A minireview by West also compared effects of noni fruit juice in collagen deposition *in-vitro and in-vivo*. Through many action mechanisms due to diverse ingredients of noni fruit, this fruit has an impact in increasing collagen synthesis and inhibit collagen matrix degradation. Consistent with this experiment, combination of these effects induces wound healing acceleration and alleviate the skin aesthetics [31].

## 5. Conclusion

Application of noni ethanol extract cream topically on incisional wounds of male wistar rats increases collagen deposition percentage significantly based on histological observation of skin tissue after twenty-eight times of application. Utilization of different concentration of noni ethanol extract in creams also shows significant increment directly proportional to the higher levels of phytochemicals in the cream.

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