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Effectiveness of Noni (*Morinda citrifolia* L.) Leaves Extract Gel as Standardized Traditional Medicine to Accelerate Oral Mucosa Wound Healing on Wistar Rats

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ABSTRACT

Wound can be described as loss of tissue integrity which is a result of pathological change or physical trauma. Deficiency of immunity and bacterial invasion, as well as poor treatment, is significant problem of wound healing and it may be a cause of chronic wounds. Noni (*Morinda citrifolia* L.) leaf is a part of plant, empirically used to heal wounds or cuts. This study aims to analyze the role of noni leaves as oral antibacterial agent against *Streptococcus mutans* and to discover the role of noni leaves extract gel on oral mucosa wound healing based on fibroblast count and angiogenesis rate. This study was conducted by *in vitro* experimental laboratory design on *Streptococcus mutans* and *in vivo* on wistar rats. On the rats, wounds were made on palatal mucosa and they were divided into four groups; positive control group without wound, negative control group without treatment, 1st treatment group which was given 10% solution of povidone iodine and 2nd treatment group given 10% noni leaves ethanol extract gel. The development of wound was examined on days 3, 7 and 14. The result showed that noni leaf extracts in 6.25, 12.5, 25, 50 and 100%, respectively concentration were not able to inhibit the growth of *Streptococcus mutans*. However, 10% ethanolic extract of noni leaf had significant impact on palatal wound healing as observed on wound healing time, wound size, angiogenesis on 7th day and fibroblast count on 3rd and 7th day. Noni leaves ethanol extract could help oral mucosal wound healing based on angiogenesis examined and fibroblast cell count compared to other group. However, possibly noni leaves is not sensitive against *Streptococcus mutans* but according to a previous study, it had inhibitory effect for *Staphylococcus aureus*.

Key words: Noni leaves, *Streptococcus mutans*, wound healing

INTRODUCTION

Wound healing is a natural process to return the skin or mucosa back to its normal state. This process consists of inflammatory phase, proliferation phase and remodeling phase. Debridement, irrigation, antibiotics and antiseptic administration has been considered to be the usual treatment for the wound. Wound healing process may be inhibited if the wound is not handled properly and could lead to complications, for example, lack or exuberant wound healing component such as collagen fibers and cells as well as excessive wound contraction (Peterson, 2003; Kumar *et al*., 2009).

Noni is a tropical plant which is distributed in Pacific, South America, Central America and South East Asia region. All parts of this plant such as fruit, leaf and seed are useful especially for food and medication. Empirically, the leaf is used as dressing for wounded skin, sprained joint and also to relieve pain or fever. Active ingredients found in the leaves are saponins, triterpens, tannins, alkaloids, glycoside iridoids and flavonoid. Among the main effects of those ingredients which related to wound healing are saponin as antibacterial agent, tannin as hemostatic and astringent, alkaloid as analgetic and flavonoid as antioxidant and anti-inflammatory agent (Wang *et al*., 2002; Nayak *et al*., 2009; Rasal *et al*., 2008).

Kumar *et al*. (2010) discovered that ethanol extract of 10 mg mL^{-1} noni leaf has antimicrobial potency against *E. coli*, *Staphylococcus aureus* and *Bacillus subtilis*. Study by Yuslianti *et al*. (2013) suggested that pure Indonesian 10% ethanol extract of noni leaf could accelerate wound healing process on the skin of Wistar rats and significantly reduced the wound size on day 3, 7 and 14, as compared to the skin treated by 10% povidone iodine (Kumar *et al*., 2010; Yuslianti *et al*., 2013).

Povidone iodine is one of the popular medications used topically on wounds and it is usually applied on skin and oral mucosa. Povidone iodine has local antibacterial effect but it could also trigger hypersensitivity reaction in some individuals, cause irritation and it has bitter taste (Gottardi, 2001). Although, the side effects were seldom being reported, it would be better if there existed another alternative for wound treatment that is more effective, safer, cheaper, easier to acquire and taste better.

In this study, we analyze the effectiveness of noni leaves as traditional medication to accelerate wound healing process by examining the clinical wound size, histopathologic findings of fibroblast cells and new blood vessel and its effect against oral bacteria *Streptococcus mutans*.

MATERIALS AND METHODS

The study was conducted at Biochemistry and Molecular Biology Laboratory of Medical Faculty General Achmad Yani University and Pharmacy Laboratory of Science Faculty General Achmad Yani University, Cimahi, West Java for the period of November 2013-January 2014. Wistar rats were utilized in this study after an ethical clearance was received from Ethical Committee of Hasan Sadikin Hospital, Bandung, West Java.

Extraction of noni leaves: Five kilograms of noni leaves collected from Cimahi, West Java were extracted into 100 g of thick ethanolic extract. An oven was used to dry the leaves, macerator was purposed to extract the leaves into light extract and the final thick ethanolic extract was done using rotary evaporator with 96% ethanol. The extraction process was performed at PAU Laboratory, Bandung Institute of Technology. Afterwards, the final extract was made into 10% gel preparation at Pharmacy Laboratory, Faculty of Science, General Achmad Yani University.

Oral antimicrobial effect examination: Ethanol extract gel of noni leaves was made into 5 different concentrations (6.25, 12.50, 25, 50 and 100%, respectively) to examined the antibacterial activity against *Streptococcus mutans*. Materials used in this study were sterilization device and disinfectant, micropipette, microbiology kit for diffusion agar method, petri dish and jars, microscope, spectrophotometer, Mueller Hinton Agar (MHA) plate, NaCl 0.9%, sterile aquadest and 50% propolis concentrate as positive control.

Oral mucosa wound examination: Wistar rats were adapted in the laboratory for 7 days before treatment. They were fed with pellets and given fresh water every day and then their weights were measured after a week. Prior to creating the wounds, rats were given 10 mL/1000 g ketamine as means of general anesthetic. The wound was made with 4 mm diameter skin/mucosal punch device on the palatal mucosa and subsequently irrigated with sterile aquadest.

Rats were made into four groups:

Group 1: Negative control group which was wounded but obtain no treatment (K1)
Group 2: Negative control group which was not wounded (K2)
Group 3: Treatment group which was wounded and given 10% noni leaf extract gel (P1)
Group 4: Treatment group which was wounded and given 10% povidone iodine solution

Noni leaf extract and povidone iodine were applied twice a day on wounded mucosa. The wounds were measured by using caliper and ruler on 3 rats from each group after they were terminated on days 0, 3, 7 and 14. Diameter of wound area was measured using a formula:

$$Dx = \frac{dx1 + dx2 + dx3 + dx4}{4}$$

where, Dx is diameter of wound on day x.

Histological sections stained with Hematoxylin-Eosin were made to examine new-formed blood vessels and fibroblast amount, taken from the palatal mucosa about 5 mm from the wounded area and 2 mm depth. Data was examined using 40x objective expansion with light microscopy. The sections were prepared at Pathological Anatomy Laboratory of Medical Faculty.

Data analysis: Data of this study was analyzed statistically with SPSS 17.0. Data found would be presented with comparison table and relation graph. For inhibitory effect against *S. mutans* bacteria, data were analyzed with Shapiro-Wilk ($p>0.05$) as normality test, Levene test for variant homogeneity and subsequently one way ANOVA test ($p\leq0.05$) for significance. If the normality test was not significant, the significance test was accomplished by non-parametric Kruskal-Wallis test.

Wound measurement and histological findings in wound healing area were observed with one-way ANOVA test ($p\leq0.05$) and Shapiro-Wilk ($p>0.05$) as normality test using SPSS software. The one-way ANOVA test was employed for normally distributed, numerical data and non-paired sample with more than two groups. Causal relation test was done with *Post hoc* test to find out if there was any significant difference between every group.

RESULTS

Effectiveness of noni leaves on inhibiting growth of oral *Streptococcus mutans*: Identification of oral bacteria *Streptococcus mutans* was conducted using gram stain method and microscopic examination (Fig. 1). The examination showed purple-stained cocci with chain and paired formation.

The activity of noni leaves extract on inhibiting *Streptococcus mutans* growth, as seen on diameter of inhibitory zone measured, were observed in concentrations subsequently 6.25, 12.50, 25, 50 and 100%, respectively.

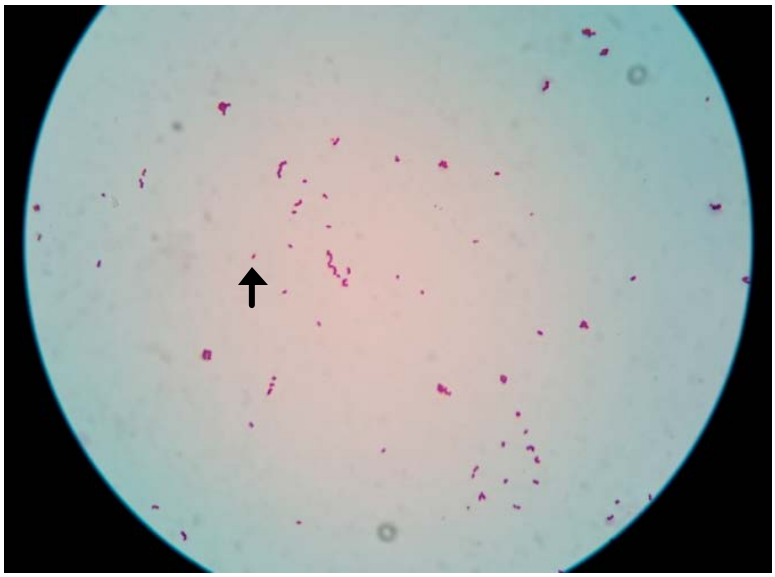

Fig. 1: Gram stained *Streptococcus mutans*

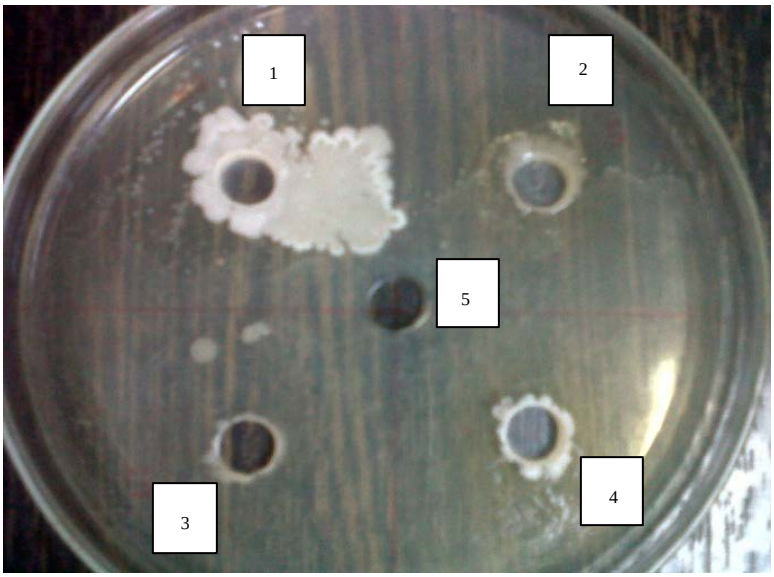

Fig. 2: Inhibitory zone formed after 18-24 h of incubation

The result of antibacterial effect examination of noni leaves to *Streptococcus mutans* showed that the inhibition zone mean was only about 0.28 mm for 100% concentration which was less than 6 mm. According to David Stout criteria, an effective inhibitory zone is supposed to be more than 6 mm width. It means that noni leaves has no effect in inhibiting growth of *Streptococcus mutans* (Fig. 2). On control plate, there weren't any bacterial growth found which means the media was not contaminated.

Table 1: Inhibitory zone of *S. mutans* by noni leaves extract

	Inhibitory zone			
Noni leaves concentration (%)	1	2	3	4
6.25	-	-	-	-
12.5	-	-	-	-
25	-	-	-	
50	-	-	-	+
100	-	-	-	-
Positive control		+		
Negative control		-		

+: Clear zone around the well, -: No clear zone around the well

Minimum inhibitory concentration of noni leaves against *Streptococcus mutans* can be seen in Table 1. Inhibitory zone diameter which was created on 6.25, 12.5 and 25%, respectively concentration showed that there wasn't any suppression on the growth of the bacteria and on 50% concentration, there was only a slight inhibitory zone seen. It could be concluded that noni leaves extract has no effect on inhibiting the growth of *Streptococcus mutans*.

Effect of noni leaves ethanol extract gel on wound healing observed on wound width and healing time

Palatal wound width on day 0: Wound width on day 0 was 4 mm on all groups, equivalent with the diameter of mucosal punch blade. The wound area appeared to be lighter by natural vasoconstriction process as hemostasis occurred on the primary phase of wound healing.

Palatal wound width on 3rd day: Wound size was reduced to 3 mm in two rats and 2 mm in one rat on noni leaves extract group. On povidone iodine group, only one rat had its wound reduced to 3 mm. However, on the group without medication, the wound diameter was still 4 mm, the same value as on day 0. The surface of mucosal wound on povidone iodine group was drier than the group without medication. A thin layer of new mucosa epithelium was seen on the palate of the rats on noni leaves extract group.

Palatal wound width on 7th day: The wound size decreased to 3 mm on one rat and on two other rats the wound diameter still measured as 4 mm. On povidone iodine group, the wound was also reduced, 3, 1 and 0 mm, respectively. The wound size was also reduced significantly on ethanol extract group.

Palatal wound width on 14th day: Two rats from noni leaves extract group on 14th day had their wounds closed perfectly but one rat from the group still had a slight 1 mm wound on the palate. On povidone iodine treatment group, two rats had the wound size decreased to 1 mm and the other one had wound 3 mm in diameter. Two of three rats from the group without medication experienced wound closure to 1 mm and on the rest of it, the wound size was 1.25 mm.

The povidone iodine treatment group had the similar wound closure pattern with the noni leaves extract group but the latter was slightly better. However, the rats on the group without medication experienced a slow wound closure. On this group, the wound closure happened on 14th day with mean value of the wound diameter of size 0.6 mm but it was not completely closed.

Data analysis of wound width: Normality test using Shapiro-Wilk assessment showed only three data had normal distribution which were wound size on 3rd day of noni leaves extract group, 7th day of povidone iodine group and 7th day of positive control group without medication. In this case, Kruskal-Wallis test was conducted ($p \leq 0.05$). The result showed that the p-value of wound size on 3rd day was $p = 0.364$; on 7th day was $p = 0.044$ and on 14th day, the p-value was $p = 0.558$. It indicated that there was a significant difference of wound size between the groups on day 7.

The result of *Post Hoc* Mann-Whitney test (Fig. 3) showed the p value between non-medication group and noni leaves extract group on day 3, 7 and 14; were $p = 0.246$, $p = 0.034$ and $p = 0.317$, respectively; comparison of the group without medication and povidone iodine group were; $p = 1.00$ on day 3, $p = 0.058$ on day 7 and $p = 0.796$ on day 14. On the other hand, between noni leaves extract group and povidone iodine group on day 3, 7 and 14, respectively, the p-values were $p = 0.254$, $p = 0.317$ and $p = 0.317$.

Based on the statistical analysis, the difference on wound size between the control group without medication and noni leaves extract group was significant on 7th day. However, comparison among the rest of the groups showed no significant results.

Effect of noni leaves ethanol extract on wound healing measured by angiogenesis and fibroblast cells around the wound area

New blood vessel formation around wound area on day 0, 3, 7 and 14: The wound were prepared on day 0 and subsequently the section was examined with light microscope. There was no sign of new blood vessel formation around the wounded area. Some red blood cells were seen among the connective tissue. Figure 4 showed the state of normal palatal mucosa tissue of Wistar rat.

Examination on the sections made on 3rd day after preparation of the wound showed that on the group without medication, the amount of visible new blood vessels on each sample were 2, 1 and 1; on povidone iodine group were 3, 2 and 5 and on noni leaves ethanol extract treatment group were 3, 5 and 7. Infiltration of inflammatory cells were obvious on each group.

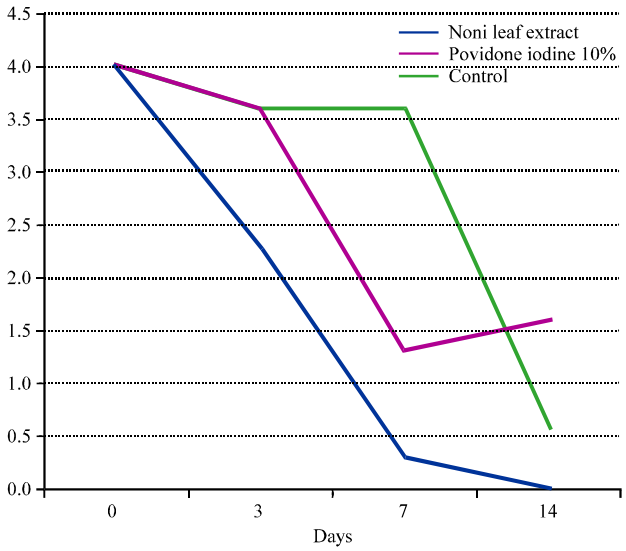

Fig. 3: Comparison of wound width on 0, 3, 7 and 14th day

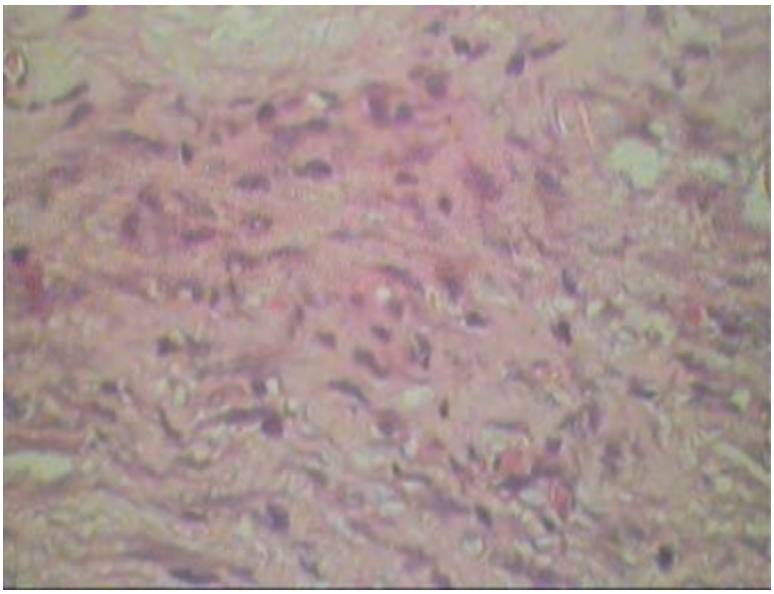

Fig. 4: Histological section on day 0 normal oral mucosa of Wistar rat

By the 7th day, wound preparations were also made on each group. The amount of visible new blood vessels on the group without treatment were 2, 3 and 3, respectively; on povidone iodine group were 2, 1 and 2 and on noni leaves ethanol extract treatment group were 13, 12 and 15, respectively (Fig. 5). New blood vessels examined on each sample for 14th day wound, were 3, 4 and 11, respectively on the group without treatment; on povidone iodine group were 4, 4 and 7 and on noni leaves ethanol extract treatment group were 25, 28 and 32, respectively.

Fibroblast cell examined around wound area on day 0, 3 and 14: Fibroblast cell amount on the day 14 was examined from three rats which had terminated and sectioned. Fibroblast cells seen here were the cells on normal connective tissue. The fibroblast cells were counted using tally counter with light microscope. The area examined on newly formed granulation tissue was near the area close to the wound made. On the group without treatment, amount of fibroblast cells was 47, 50 and 61, respectively. On povidone iodine group there were 80, 82 and 98, respectively. On noni leaves extract group, the amount was 95, 69 and 85, respectively. Inflammatory cells were seen on each group (Fig. 6).

The amount of fibroblast cells found on each section was 68, 57 and 87, respectively on non-treated group, on povidone iodine group was 87, 82 and 115 and on noni leaves ethanol extract treatment group were 110, 132 and 118, respectively.

Fibroblast cell examined on each sample on 14th day, on the non-treatment group were 87, 55 and 82, respectively on povidone iodine group were 55, 104 and 114 and on noni leaves ethanol extract treatment group were 146, 118 and 114, respectively. Inflammatory cells were still prominent on the group without any medication and new connective tissue formation was dense in the group treated with noni leaves extract (Fig. 7).

Statistical analysis on blood vessel formation and fibroblast count: Normality test on blood vessel formation examined showed that all groups had significancy, therefore,

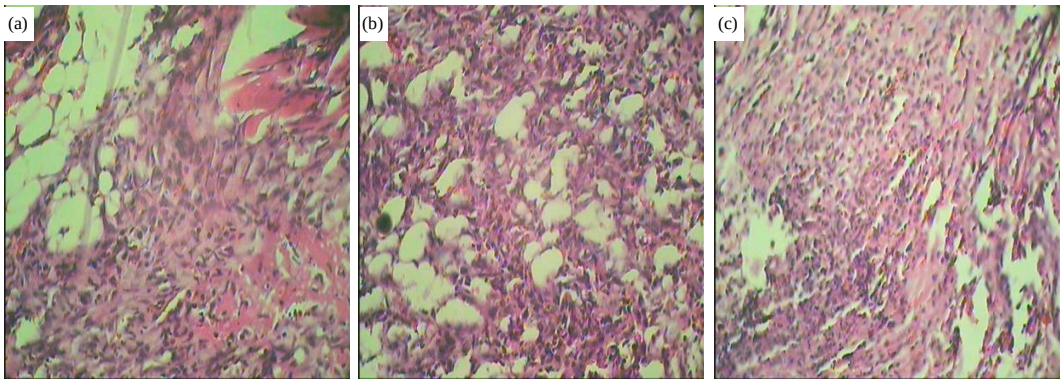

Fig. 5(a-c): Histological appearance of wound area on day 7 (a) Positive control group, (b) Povidone iodine and (c) Noni leaves extract

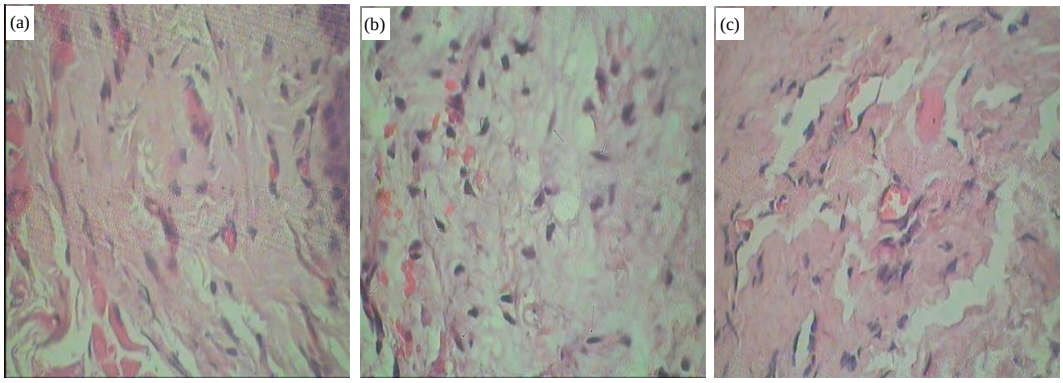

Fig. 6(a-c): Microscopy of wound area on day 3 (a) Control group, (b) Povidone iodine group and (c) Noni leaves extract group

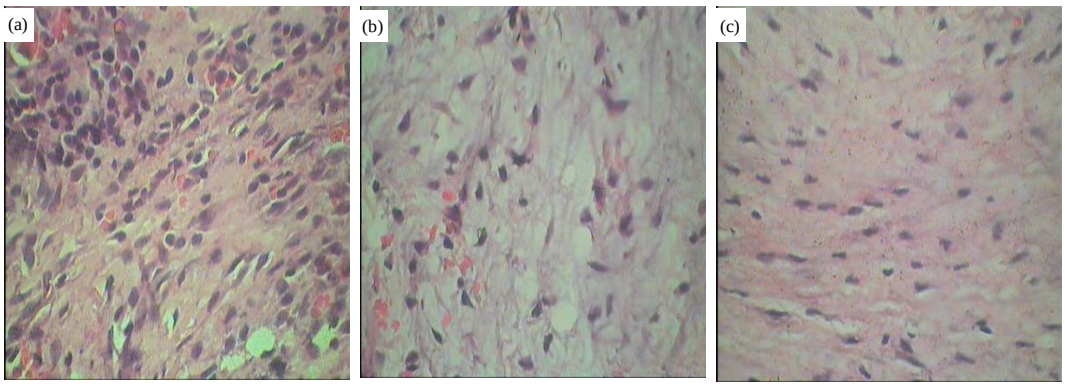

Fig. 7(a-c): Histological appearances on wound day 14 (a) Positive control group, (b) Povidone iodine group and (c) Noni leaves group

Table 2: Statistical analysis of blood vessel and fibroblast cell count

	p-value (one-way ANOVA test)	
Days	New blood vessels	Fibroblast count
3	0.024*	0.013*
7	0.000*	0.019*
14	0.008*	0.083

*Statistically significant

Table 3: *Post hoc* tukey test result of blood vessel count

Days	Noni leaves (control)	Povidone iodine (control)	Povidone iodine-noni leaves
3	0.022*	0.093	0.500
7	0.001*	0.306	0.000*
14	0.010*	0.843	0.018*

*Statistically significant

Table 4: *Post hoc* tukey test result of fibroblast cell count

Days	Noni leaves (control)	Povidone iodine (control)	Povidone iodine-noni leaves
3	0.027*	0.017*	0.904
7	0.216	0.201	0.175
14	0.679	0.077	0.231

*Statistically significant

one-way ANOVA test ($p \leq 0.05$) was performed and the result displayed that blood vessel count on day 3, 7 and 14, respectively were significant ($p = 0.024$, $p = 0.000$ and $p = 0.008$).

One-way ANOVA test ($p \leq 0.05$) was also performed on fibroblast cell count since the normality test using Shapiro-Wilk showed normal distribution on every group. On day 3, 7 and 14 examination, the statistical analysis result demonstrated that all result were not statistically significant, in which the p-value for 3, 7 and 14th day, respectively were $p = 0.013$, $p = 0.019$ and $p = 0.083$ (Table 2).

Post hoc Tukey test result for each groups showed there was significant difference on blood vessel formation count on day 7 and 14 between noni leaves extract group with both control and povidone iodine group. There was no significant difference of blood vessel count between control group and the group given povidone iodine (Table 3).

However, *Post hoc* Tukey test conducted on the data of fibroblast cells showed no statistical significance on every other group mostly, except between control group with two other group on 3rd day (Table 4), although the numerical result showed a difference and the statistical analysis was significant on day 3 and 7 (Table 2).

DISCUSSION

Noni leaves ethanol extract gel has a benefit on oral mucosa wound healing possibly because of the active ingredients. Scopoletin, one of the active constituent, has an anti-inflammatory effect and has an ability to help forming the granulation tissue during the healing process. Antioxidant elements inside the leaves such as glutamic acid, carotenoid and flavonoid are able to inhibit the release of Reactive Oxygen Species (ROS) which damages the connective tissue and its cells so that the new connective tissue formed during late inflammatory phase can be maintained. Glutamic acid and carotenoid that have a role on preserving the immune system. Antibacterial agents like xeronine, proxeronine, saponin, ursolic acid and anthraquinon also affect the inflammatory process

through antimicrobial effect, they prevent bacteria around the wound area to invade it. Xeronine and proxeronine work by inhibiting peptidoglycan construction of bacterial wall and encouraging lysis of the bacteria. Saponin acts as an agent to stop bacterial cell membrane function while, antraquinon inhibits bacterial synthesis. The debridement effect, created by the interaction of noni leaves extract with the wound, produce enzymatic activity of protease which can help in clear necrotic debris, infected or damaged tissue to promote wound healing (Enoch *et al*., 2008; Middleton *et al*., 2000; Nayak *et al*., 2009; Lipsky and Hoey, 2009; Rasal *et al*., 2008).

Based on the result of clinical wound examination, it can be assumed that noni leaves extract has a potency to help the wound to heal faster and without complication. The extract helped wound healed on day 3, 7 and 14 as compared to application of povidone iodine treatment and without treatment. It also meant that noni leaves extract offers as a medication on every phase of wound repair. Blood vessel formation on the newly-formed tissue as one of the indicator of neoangiogenesis was slightly higher in the group treated with noni leaves extract, although it was not statistically significant. Possibly, antiseptic, anti-inflammatory and antibacterial effect of the active constituents of the leaves was responsible in this process. The new blood vessels formed can provide nutrition better for the new tissue so it helped the healing process (Kumar *et al*., 2009; DiPietro and Burns, 2003).

The amount of fibroblast can be a marker for wound healing process especially on excisional wounds that require connective tissue recovery. Newly formed fibroblast by activation of growth factors such as TGF-β and FGF from macrophage to the rest of fibroblast progenitor in the connective tissue are appeared by the end of inflammation phase to the remodeling phase. Based on the examination, the wound treated by noni leaves extract had slightly higher fibroblast count as compared to povidone iodine-treated group and more significant compared to the group without treatment, although statistically the difference was not significant. One of the active ingredients worked in this process is possibly flavonoid by avoiding degradation of connective tissue cells by ROS which usually take part on wound healing and inflammation process (Eming *et al*., 2007; Kumar *et al*., 2009).

In this study, it had found that noni leaves extract did not succeed to inhibit the growth of bacteria *Streptococcus mutans* which is the normal flora found inside the oral cavity. Probably, the antimicrobial agents found on the leaves (saponin, alkaloid and ursolic acid) was more active to other bacteria specifically such as *Staphylococcus aureus* (Kumar *et al*., 2010), which can be found on cutaneous wounds (Kumar *et al*., 2010; Philip and Martin, 2009).

CONCLUSION

It can be concluded that noni leaf didn't have an inhibitory potential against bacteria *Streptococcus mutans* on all concentration assessed. However, 10% topical noni leaves extract gel, compared to 10% povidone iodine proved to help wound repair by reducing wound size on oral mucosa and accelerating the healing process clinically, increasing the amount of fibroblast cells and helped the angiogenesis process as examined on of new blood vessels formed during the wound healing phases.

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