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ORIGINAL ARTICLE

Benefits of Ethanol Based Noni Leaf (*Morinda citrifolia L.*) Extract on Oral Mucosal Wound Healing by Examination of Fibroblast Cells

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ABSTRACT

Wound healing is a biological response that occurs following a trauma or pathologic condition of the oral mucosa. Wounds require proper management so they may heal faster and without complication. The number of fibroblast cells seen in the oral mucosa is a strong indicator of wound healing. In the remodelling phase of wound healing, fibroblast cell proliferation decreases as collagen fibres are synthesized. Noni leaf (*Morinda citrifolia L.*), a part of the noni plant, is traditionally used to heal soft tissue wounds in Indonesia. The leaves have potential chemical compounds that may be useful in the wound repair process. **Objective:** This paper aims to examine the effect of ethanol based pastes of the Indonesian noni leaf on oral mucosa wound healing by investigating visual wound closure and fibroblast cell counts in Wistar rats. **Methods:** Rats were divided into 2 control groups and 4 treatment groups. The pastes were formulated in concentrations of 2.5%, 5%, 10%, and 20% and were applied onto wounds of the oral mucosa of the rats. One control group was given no medication and the other control group had an ethanol gel with a concentration of 10% noni leaf extract applied to their intraoral wounds. **Result:** Result revealed that all groups treated with noni leaf paste experienced better wound closure ($p < 0.05$) when compared to the control groups. Fibroblast cell counts showed little significance amongst all groups ($p = 0.143$), however fibroblast cell counts of groups treated with noni leaf paste, of all concentrations, were lower than both control groups. **Conclusion:** These results suggest that topical application of noni leaf paste promotes better oral mucosa wound healing than gel formulas but shows no significant difference in the amounts of fibroblasts during the remodelling phase.

Keywords: fibroblast, noni leaf, paste, wound healing.

INTRODUCTION

Oral mucosa wounds are a reaction to trauma or pathological conditions with varied aetiological factors, for example traumatic lesions or aphthous stomatitis.^{1,2} Intraoral wounds can cause discomfort and a reduction in normal function. The human body has a natural healing response to skin and mucosal wounds that occurs in three phases; the inflammatory, proliferative, and remodelling phases. If wounds are not managed properly certain aspects of the phases may be prolonged. Therefore, correct management of the wound is essential to optimal healing in a short time.¹⁻³

Fibroblasts are a significant cell of the oral mucosal connective tissue and their main function is to synthesise collagen, elastic fibres, and extracellular

matrix or connective tissue ground substances, such as glycosaminoglycans and proteoglycans.⁴ Fibroblasts can be derived from bone marrow stem cells, epithelial cells, endothelial cells, pericytes, or vascular smooth muscle.⁴ During the wound healing process, damage to connective tissue stimulates chemotaxis and proliferation of fibroblasts to repair the injured area of tissue.⁵ This process is facilitated by mediators such as platelet-derived growth factor, fibroblast growth factor, transforming growth factor- β , and interleukin 6, which are synthesised by cells in the extracellular matrix. Fibroblast proliferation slows toward the end of the proliferative phase and in the beginning of the remodelling phase of wound healing.⁶ As the connective tissue returns to its healthy state, fibroblasts differentiate into their quiescent form, known as fibrocytes. Fibrocytes are smaller, spindle shaped cells

with finer cytoplasm and are less active in producing collagen.⁴

Indonesian noni (*Morinda citrifolia* L.), known locally as *mengkudu* or *pace*, is a tropical plant that is tolerant to various weather conditions and can thrive in a wide range of habitats. The noni plant can be found in many regions of Indonesia, and has a unique concentration of active constituents when compared to noni plants found in other parts of the world.⁷ Historically the leaves of the noni plant were used to heal boils or wounds by grinding it to a paste and applying it to the lesion.⁸ Researchers have identified many active components within the leaves including flavonoids, saponins, tannins, glycosides, scopoletin, anthraquinones, amino acids, sterols, coumarins, triterpenoids, and alkaloids. Some of these compounds have proven benefits in wound healing promotion, such as the flavonoids, tannins, anthraquinones, alkaloids, glycosides, and scopoletins, which have antioxidant, astringent, antiseptic, and antibacterial qualities.⁷⁻¹⁰

Research by Nayak and Rasal showed that the oral administration of noni leaf extract can promote wound healing in the skin of Wistar rats.^{9,10} Further studies that analysed wound closure, fibroblast cell counts, neovascularisation, and inflammatory cell counts have found that the topical application of noni leaf extract can also stimulate wound healing in the oral mucosa and skin of Wistar rats.¹¹ A research paper by Kumar, that examined the inhibition zones formed on agar, also found that ethanol based noni leaf extract has an inhibitory effect on *Staphylococcus aureus*, a common bacteria found in skin wounds.¹³ A previous study carried out by the authors of this paper, showed that Indonesian noni leaf extract contained flavonoid compounds and a topical ethanol based extract gel in 10% concentration was effective on both skin and oral mucosa wound healing. This observation was noted by measuring the width of the wound, fibroblast counts, and antioxidant activity.^{13,14} This study aims to utilise noni leaves as a topical medication for wounds in the oral mucosa and examine which concentration promotes greater wound healing, particularly during the remodelling phase. The hope is to develop a more efficient formula with low toxicity, in the form of paste, which is more resistant to oral fluids and has a protective effect on the wound surface.

METHODS

Research was carried out between August and November 2015 at the Animal and Pathology Anatomy Laboratories of the Medical Faculty, General Achmad Yani University, Cimahi, West Java. 30 Wistar rats were used after ethical clearance was obtained from the Hasan Sadikin General Hospital in Bandung. The noni leaves used were harvested in Unjani, Cimahi in

West Java according to GACP and were identified in the Bandung Institute of Technology. The leaves were processed to adhere with the herbal medicine standard from the Pharmacy Laboratory of the Science Faculty, General Achmad Yani University, Cimahi, West Java.

Wound healing model

The palatal mucosa of 30 Wistar rats (*Rattus norvegicus*) was used in this study to examine fibroblast proliferation and reduction of wound size throughout the wound healing process.^{16,17} The subjects were placed into a state of general anaesthesia by administration of 10 ml/100 g ketamine via intraperitoneal injection on the posterior of their left lateral abdomen. Wounds measuring 4mm in diameter and 2mm in depth were then made using a punch biopsy device on the palatal mucosa of the rats. Following wound procedures, rats were then divided into 6 groups with 5 rats in each group. Groups were categorised as follows; control group that received no topical applications, control group that received noni leaf ethanol extract in a gel formula, and four groups that received noni leaf pastes of differing concentrations (2.5%, 5%, 10%, and 20% respectively).

Ethanol-based extract gel and paste formula

The noni leaves were macerated to obtain ethanol based extracts which were then processed into gel and paste formulas. Ethanol extracts of the noni leaf was made by desiccating 5 kg of shredded leaves that was then placed into an oven at 50 °C for 2 days. Following the desiccation process, the remaining 1 kg of semisolid leaves were refined into powder form and made into ethanol based extracts, using 96% ethanol. The powder was then placed into a macerator for 24 hours until all the powder had become liquid extract. A rotary evaporator was subsequently used to evaporate the excess liquid to thicken the extract, which resulted in a thick, dark green substance. This concentrated ethanol extract of noni leaf was then formulated into a 10% gel extract by adding 100 g of gel base for every 10 g of concentrated extract.¹⁸ The noni leaf paste was formulated by mixing the same concentrated ethanol extract with a base formula that consisted of 20% gelatine, 15% zinc oxide paste, 2.5 mg glycerine, 0.5% sodium benzoate, and water using a spatula to achieve the desired concentrations.¹⁸ As an example, a paste with a concentration of 2.5% of noni leaf extract was achieved by mixing 100 mg of base and 2.5 mg of extract, with pastes of differing concentrations made using the same technique.

Wound width examination

The ethanol based noni leaf extract gel and pastes were applied to each group's wounds, using wooden spatulas, two times a day for 14 days. On the 1st, 3rd, 7th, and 14th day wound diameter was measured using sliding calipers. Measurements of the wounds of each rat was recorded and calculated using the following formula;

Table 1. Mean values of wound width (in mm)

Groups	Mean
Control without intervention	1
Control with 10% gel application	0.4
2.5% paste application	0
5% paste application	0
10% paste application	0
20% paste application	0

Table 2. Analysis of mucosal healing by wound width measurements on day 3, 7, 14

Examination day	p value
Day 3	0.07
Day 7	0.00*
Day 14	0.00*

*statistically significant (p<0.05)

Table 3. Mean value of fibroblast cell count

Groups	Mean value
Control without application	94.28
Control with 10% gel application	85.56
2.5% paste application	64.02
5% paste application	69.88
10% paste application	75.84
20% paste application	70.48

final diameter = (d0+d3+d7+d14)/4, in which d0 was the wound diameter on day 0, d3 was the diameter on day 3, and so forth.

Fibroblast cell examination

Microscopic examination of fibroblast cells was performed on the wound tissue on the 14th day, which was the end of the proliferative phase and the beginning of the remodelling phase. Wound tissue was excised, with a 1 mm border, from the palatal tissue of all subjects and was placed onto slides for examination. These tissue sections were then processed and stained using the standard Hematoxylin and Eosin procedure. The fibroblast cells were examined and counted using light microscopy (40x objective) on 5 random visual planes for every group.

Data analysis

Fibroblast cell counts for all groups were analysed using the ANOVA test, followed by Tukey post hoc tests, p values less than 0.05 were considered significant. Prior to the data analysis, normality testing (p > 0.05) was performed using the Kolmogorov-Smirnov test. If the data was abnormal, a Kruskal-Wallis test followed by the Mann-Whitney test as a comparative assessment was performed.

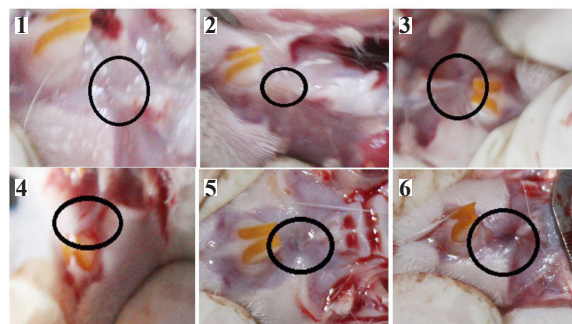


Figure 1. Wound closure on day 14 showing each group including the control group (1), the gel group (2), 2.5%, 5%, 10%, and 20% paste concentration (3-6 respectively).

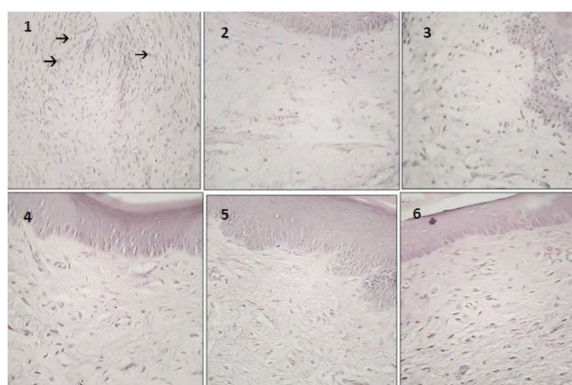


Figure 2. Histopathological features of each group under 400x magnification; 1) Control group without medication; 2) Control group with using ethanol extract gel; 3) Group with 2.5% paste concentration; 4) with 5%, 5) with 10%, and 6) with 20% concentration. The inflammatory cells are still visible in the control group without medication (arrow sign), whilst fibroblasts can be seen in every group.

RESULTS

Wound closure examination

All subjects in the noni leaf extract paste groups showed better wound healing than the control groups when examined on days 3, 7, and 14 following the initial injury. In the control group that was given no intervention, the average wound width diameter was 1 mm, whilst the group that received noni leaf in a gel extract had a mean wound diameter of 0.4 mm. In contrast, all groups treated with noni leaf extract pastes, at every concentration, had mean wound diameters of 0 mm, or complete closure without a visible wound or depression.

A Kruskal-Wallis test was performed on the data relating to wound width, as the data distribution was abnormal (Table 2). The test results (p < 0.05) on width measurements of the wounds showed a significant difference amongst all groups (p = 0.00). Mann-Whitney comparative tests showed that the most significant differences were between the control group

without any intervention and the group who received a paste with a 2.5% concentration, and between the same control group and the group who received a paste with a 20% concentration ($p = 0.031$).

Microscopy and fibroblast cell examination

Microscopic examination of the healing wounds showed fibroblast cells in all control and treatment groups (Figure 1). In the control group several inflammatory cells were still visible, whilst in the groups who received treatment some fibrocytes were identified. The highest mean value of fibroblasts was found in the control group not receiving any topical applications, and the lowest count was in the group receiving the 2.5% extract paste, results are shown in Table 3.

Kolmogorov-Smirnov tests carried out for the data distribution was normal. The results of ANOVA testing ($p < 0.05$) on fibroblast cell counts showed no significant difference between all groups ($p = 0.143$). There was a small decrease in the amount of fibroblasts in the groups receiving noni leaf extract pastes but the data was not significant amongst the groups.

DISCUSSION

This study found that wound healing in the oral mucosa can be promoted with the use of topical medications. Topical antiseptics have been used for this purpose, however they can have side effects such as irritation of the mucosa.^{14,15} Topical formulas that can be applied to oral mucosal lesions are pastes, which are relatively resistant to saliva and oral fluids offering a protective effect.¹⁹ Previous studies have found that topical herbal formulas taste better than conventional antiseptics when applied to the oral mucosa.²⁰ This study found that ethanol based extract of the noni leaf in a paste formula may help excisional wounds of the oral mucosa repair faster by means of wound width and fibroblast cell quantity.

The remodelling phase of excisional wound healing usually occurs after day 14. This phase is characterised by new epithelium that forms a new skin or mucosal surface, rearrangement of collagen fibres, and clinically the wound has started to close.⁶ Results from this study revealed that mucosal wounds healed faster when the noni leaf extract was administered as a paste. This is a similar result to the Author's previous study where an ethanol based gel with a noni leaf concentration of 10% was found to hasten oral mucosal wound healing.¹⁴ In normal wound healing the newly formed connective tissue fills the previously wounded area and fibroblast cells return to their dormant fibrocyte form.⁴ In this study when fibroblast cell counts were performed under microscope, the amount of fibroblasts in the control

group was higher than all treatment groups, although it was not statistically significant. This demonstrates that fibroblast cell proliferation in all treatment groups had reduced earlier than in the control group, which indicates that noni leaf pastes can help wounds to heal faster.^{3,21}

Many active constituents of noni leaf extract are believed to promote faster wound healing and closure and accelerate fibroblast cell maturation. Saponin, for example, has antibacterial properties and may lower the risk of infection. Tannins are known astringents and can help with haemostasis following an injury, however it does not specifically affect fibroblasts in the remodelling phase.⁹ Iridoid glycosides and flavonoids found in noni leaves have antioxidant properties, which eliminate free radicals and unpaired oxygen that may cause cell damage. Flavonoids may also inhibit MMP synthesis indirectly by inhibiting TNF- α expression in macrophages, which can also cause tissue damage and therefore slow the wound repair process including prolonging the proliferation phase.^{6,22}

The preparation and doses of active ingredients in medications will influence the effectiveness of the medicine.²³ Although statistical tests on fibroblast cell counts were not significant in this study, if we look at the mean values of fibroblast counts and wound closure, a paste formula of noni leaf extract is more effective in promoting faster wound healing than a gel formula. Pastes are known to be more resistant to oral fluids and saliva, and they have protective properties to the oral mucosal surface.¹⁹ Fibroblast cell count examination revealed that an ethanol based noni leaf extract paste in a 2.5% concentration was better than other concentrations in acceleration of wound healing and fibroblast maturation. These results also show that efficacy of a medication is not always directly proportional to its concentration of active ingredients.²³

CONCLUSION

This study demonstrated that whilst ethanol based noni leaf extracts in a paste form has little effect on fibroblast cell counts in the remodelling phase, it is effective in accelerating the healing process according to clinical wound closure measurements. Wound healing is a complex process that is influenced by many biological aspects, such as inflammatory cells, vascularization, and growth factors. Whilst noni leaf extract was not found to influence fibroblast cell count during the remodelling phase it may affect these other factors. In further studies we will examine fibroblast cell counts in all wound healing phases and perform an immunohistochemistry analysis of collagen fibres as a possible important indicator of wound repair.

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