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Original Article

Effect of *Clinacanthus nutans* Leaf Extract on Oral Mucosal Burns and Tongue Wounds: An *In-vivo* Study

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ABSTRACT

Background: *Clinacanthus nutans* exhibits antioxidant, anti-inflammatory, antiviral, anticancer, and anti-biofilm activities. Wounds in the oral cavity can affect the quality of life. Wound healing is a complex process to restore functions of injured tissues. The effect of *C. nutans* on oral mucosal burns and tongue wounds of rats is investigated. **Methods:** Forty male Sprague Dawley rats were divided into negative control and treatment groups with 25, 50, and 100 mg/mL of *C. nutans*. Oral mucosal burns and tongue wounds were observed on days 7 and 14 based on the fibroblast count and collagen deposits. Data were analyzed by analysis of variance and *post hoc* Tukey's test with $P < 0.05$. **Results:** Results revealed that on day 7, the fibroblast count of mucosal burns and tongue wounds increases. The maximum mucosal burns were observed for the 100 mg/mL group, whereas the maximum tongue wounds were observed for the 25 mg/mL group. On day 7, collagen deposits were increased in case of mucosal burns. The number of collagen deposits increased on day 14, the highest mucosal burns were observed for the 100 mg/mL group, and in case of tongue wounds, the highest number was observed for the 50 mg/mL group. **Conclusion:** Ethanol extracts of *C. nutans* leaves can increase the fibroblast count on day 7 and collagen deposits on day 14 after injury in case of oral mucosal burns and tongue wounds during the wound healing of Sprague Dawley rats.

KEYWORDS: *Clinacanthus nutans, oral mucosal burns, tongue wound, wound healing*

BACKGROUND

Burns constitute damage or loss of tissue caused by contact with heat sources such as fire, hot water, chemicals, electricity, and radiation.[1] Burns that are caused by high temperature in the oral cavity penetrate to vesicular or ulcerative lesions. Clinically, the appearance of burns depends on the severity and destruction of tissues; sometimes, nerve fibers are affected.[2] Most of the thermal lesions in the mouth are related to burns caused by hot food.[3] Management in the healing of burns, including prevention of infection, provides an opportunity for the remnants of epithelial cells to proliferate and close the wound surface. Antibiotics are typically prescribed for preoperative surgical prophylaxis in oral burns.[4]

The tongue is an important organ that plays a role in communication, food taste, chewing, and swallowing. If there are wounds on the tongue, the wounds affect the quality of life of people. Tongue lacerations can result from self-harm, seizures, blunt force facial trauma, oral trauma, and child abuse.[5] Wound healing is a complex

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and dynamic process that aims to restore the integrity and function of the injured tissues through several stages, viz., hemostasis, inflammation, proliferation, and remodeling.[6] After injury, hemostasis response occurs at the wound site to prevent blood loss. Inflammatory cells are attracted to the wound site to remove bacteria and foreign objects.[6-8] At the proliferation stage, re-epithelialization occurs, accompanied by neovascularization. Fibroblast synthesizes and secretes extracellular matrix (ECM) proteins and releases growth factors.[8] Eight days after injury, remodeling begins and continues for about a year.[9]

As a plant that grows in Southeast Asia, *Clinacanthus nutans* leaves extract (CNE) has been used already in traditional medicine as a drug to cure dysentery,[10] snake bites, herpes simplex virus, and varicella zoster virus lesions,[11] among others. The *in-vitro* study of the chloroform extract of this leaf has been reported to demonstrate potential for oral wound healing, as well as anti-inflammatory, anti-biofilm,[12] anti-oxidant,[13] anticancer,[14] antibacterial,[15] and antiviral effects.[11] *C. nutans* contains flavonoids, phenolic compounds, saponins, diterpenes, and phytosterols.[15] Flavonoids play a role in healing burns because these compounds can inhibit the growth of bacteria in living tissues; flavonoids exhibit anti-inflammatory activity as well as increased fibroblast proliferation.[12,16] A previous study has reported that the chloroform extract of CNE can accelerate the migration of human gingival fibroblasts *in vitro*.[12] Besides flavonoids, saponins also can induce angiogenesis.[17] These activities are required to support wound healing. The aim of this study is to investigate the effect of CNE on burns in oral mucosa and tongue wound healing.

Materials and Methods

This study was conducted at the Biocore Laboratory and Opadcore Laboratory, Faculty of Dentistry, Trisakti University, Jakarta and at Molecular Biology and Proteomics Core Facilities, Faculty of Medicine, Universitas Indonesia, Jakarta, Indonesia. Ethical approval for this study (0965/UN2.F1/ETIK/2018) was provided by the Research Ethics Committee Faculty of Medicine, Universitas Indonesia, Jakarta, on September 17, 2018.

Plant material preparation

First, *C. nutans* leaves were washed thoroughly using water to remove the attached dust particles. Next, the leaves were dried for approximately 2 weeks. The dried leaves were ground to a powder using a blender. One hundred grams of the *C. nutans* powder was macerated using 500 mL of ethanol for 72 h. Then, the extract residue was filtered and concentrated using a rotary evaporator at 50°C. This procedure was repeated three times. The water solubility of the ethanol extracts was increased by mixing them with polyvinylpyrrolidone (Sigma-Aldrich, St Louis, MO, USA) in a 1:10 ratio (w/w), and the resulting solutions were used for treatment to animals. The obtained ethanol extracts were stored at −20°C until the extract was ready for use. The *C. nutans* extracts (CNE) were mixed with Orabase before application to samples.

Animal samples

Forty male Sprague Dawley rats were obtained from the Animal Maintenance Unit of the Faculty of Veterinary Medicine, Bogor Agricultural University, Bogor, aged 2–3 months with a body weight of 200–300 g and healthy. Before the experiments, the rats were made to adapt in laboratory cages of animals for 7 days under ambient conditions (temperature 22 ± 3°C, relative humidity of 30–70%, dark–light conditions, during each 12 h, not noisy). Prior to forming wounds, the rats were anesthetized by using a combination of ketamine and xylazine (1:1) in a general dose of 10 mL/1000 g (0.1–0.2 mL). Rats were intraperitoneally injected at 2/3 posterior from the dextral abdomen. Rats were randomly divided into eight groups, each consisting of five rats, as follows: Group 1: control (untreated), 7 days; Group 2: CNE 25 mg/mL treatment, 7 days; Group 3: CNE 50 mg/mL treatment, 7 days; Group 4: CNE 100 mg/mL treatment, 7 days; Group 5: control (untreated), 14 days; Group 6: CNE 25 mg/mL treatment, 14 days; Group 7: CNE 50 mg/mL treatment, 14 days; Group 8: CNE 100 mg/mL treatment, 14 days. Seven days implies that the samples are killed after 7 days, whereas 14 days implies that the samples are killed after 14 days. Rats before and after treatments were weighed. Burns on the oral mucosa of rats were made using a heated amalgam stopper and allowed to stand for 40 s, whereas wounds on the tongue of rats were made using a lancet, with a depth and length of 1 mm and 0.5 cm, respectively.

Specimen preparation

Prior to organ harvesting, rats were anesthetized using a combination of ketamine and xylazine. Once the rat was deeply anesthetized, the oral mucosa and tongue where the wound was made were harvested, and the organs were cleaned using physiological saline. Histology preparations of oral mucosa and tongue tissue with a diameter of 10 mm from the wound area and a depth of 2 mm were fixed using 10% normal buffered formalin. Then, the tissue was processed and cultivated in paraffin blocks. Paraffin blocks were cut with a thickness of 5 μm and placed on a glass slide.

Deparaffinization and dehydration were performed on the tissue that was on the object glass, followed by staining with hematoxylin and eosin (HE) and Masson's trichrome. Fibroblasts were counted using an expansion light microscope with a 40× objective, and collagen deposits were observed by ImageJ (Bethesda, MD, USA) software.

Statistical analysis

The statistical significance of differences among groups was assessed by two-way analysis of variance (ANOVA) and Tukey's test using GraphPad Prism version 8.2 (San Diego, CA, USA). A value of $P < 0.05$ was considered to be significantly different.

Results

Effect of CNE on oral mucosal burns based on fibroblast count

The fibroblasts for all groups were counted, and then observations and counts for days 7 and 14 were performed [Figures 1 and 2]. The mean and standard deviation of the fibroblast cell count sequentially at day 7 were 104.6 ± 21.90, 112.2 ± 14.54, 114.4 ± 14.37, 114.4 ± 12.92 and on day 14, the corresponding values were 105.0 ± 18.17, 84.16 ± 18.44, 85.12 ± 10.69, and 56.56 ± 11.38. Results obtained from the normality test by the Shapiro–Wilk test revealed that all data exhibit a normal distribution. Two-way ANOVA test results revealed significant difference ($P < 0.05$). Tukey's *post hoc* test revealed that the control group and 100 mg/mL treatment group on day 14 exhibit a significant difference.

Effect of CNE on oral mucosal burns based on collagen deposits

The number of collagen deposits in all groups was counted, and then observations on days 7 and 14 were performed [Figures 3 and 4]. The mean and standard deviation of collagen deposits sequentially on day 7 were 41.51 ± 3.977%, 47.15 ± 7.239%, 43.52 ± 6.277%, 46.91 ± 2.852%, and the corresponding values on day 14 were 41.79 ± 7.879%, 45.58 ± 4.132%, 45.12 ± 4.553%, and 55.26 ± 5.791%. Results obtained from the normality test by the Shapiro–Wilk test revealed that data exhibit a normal distribution. Two-way ANOVA test results revealed a significant difference ($P < 0.05$). Tukey's *post hoc* test revealed that the control group and 100 mg/mL treatment group on day 14 exhibit a significant difference.

Effect of CNE on tongue wound based on fibroblast count

The fibroblasts for all groups were counted, and observations and counts were performed on days 7 and 14 [Figures 5 and 6]. The mean and standard deviation of the fibroblast cell amount sequentially on day 7 were 87.00 ± 14.93, 87.67 ± 14.05, 108.5 ± 7.047, 98.60 ± 11.59, and the corresponding values on day 14 were 103.8 ± 11.39, 75.80 ± 11.67, 65.40 ± 13.35, and 54.20 ± 8.198, respectively. Results obtained from the normality test by the Shapiro–Wilk test revealed that

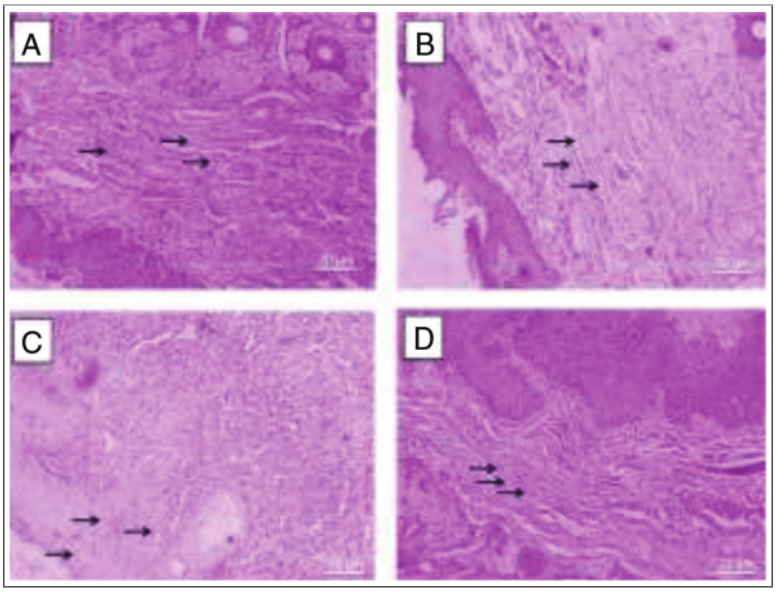

Figure 1: Representative images of the histological view of fibroblasts (black arrow) on the mucosal burn of rat tissues on day 7, which are stained with HE. A. Control (untreated). B. 25 mg/mL of CNE. C. 50 mg/mL of CNE. D. 100 mg/mL of CNE. Original magnification 40×

all data exhibit normal distribution. Two-way ANOVA test results revealed a significant difference ($P < 0.05$). Tukey's *post hoc* test revealed that between control and all treatment groups, the 50 and 100 mg/mL treatment groups on day 14 exhibit a significant difference.

Effect of CNE on tongue wound based on collagen deposits

Collagen deposits for all groups were counted, and observations on days 7 and 14 were made [Figures 7 and 8]. The mean and standard deviation of collagen deposits sequentially on day 7 were 43.31 ± 1.971%, 42.85 ± 2.620%, 40.13 ± 1.757%, and 39.12 ± 3.091%, and the corresponding values for day 14 were 40.61 ± 1.271%, 51.71 ± 1.323%, 53.03 ± 2.810%, and 46.15 ± 2.363%. Results obtained for the normality test by the Shapiro–Wilk test revealed that all data exhibit a normal distribution. Two-way ANOVA test results revealed a significant difference ($P < 0.05$). Tukey's *post hoc* test revealed a significant difference between the control and 100 mg/mL treatment groups on day 7, control and all treatment groups, 50 and 100 mg/mL groups on day 14.

Discussion

Injury constitutes tissue damage to the skin that can be caused by thermal and physical conditions, as well as changes in physiological conditions. Wound healing is a physiological process that occurs in the reactions and interactions between cells and mediators.[18] Wound healing is categorized into four processes: hemostatic, inflammatory, proliferation, and maturation stages (remodeling).[6]

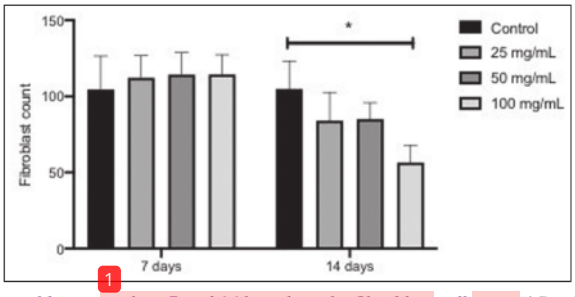

Figure 2: Effect of CNE on oral mucosal burns on days 7 and 14 based on the fibroblast cell count. *$P < 0.05$ between groups. Data are means ± SD, $n = 5$

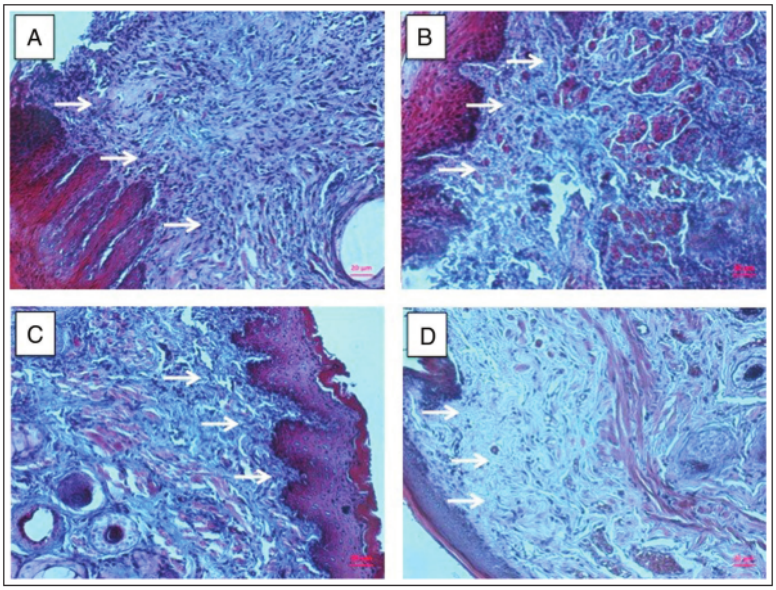

Figure 3: Representative images of the histological view of collagen deposits (white arrow) on mucosal burns of rat tissues on day 14, stained with Masson's trichrome. A. Control (untreated). B. 25 mg/mL of CNE. C. 50 mg/mL of CNE. D. 100 mg/mL of CNE. Original magnification 40×

Fibroblasts are predominant cells in the granulation tissue at the proliferation stage of wound healing.[19] Fibroblasts arrive at the injury site 24–48 h after injury,[20] which reached a peak on day 7 after injury.[21] Hence, in this study, observations were made on day 7. Although there was no significant difference, the fibroblast count for the treatment groups was greater than that in the control group on day 7 for both oral mucosal burn and tongue wound experiments. In case of the oral mucosal burn experiment, the highest number of fibroblasts was observed for the 100 mg/mL treatment group, whereas for the tongue wound experiment, the highest number of fibroblasts was observed for the 50 mg/mL treatment group. Once fibroblast infiltrates the wound site, it starts to degrade the fibrin clot by producing matrix metalloproteinases (MMPs) and replacing it with glycoproteins, collagen I–IV, heparan sulfate, proteoglycans, laminin, thrombospondin, hyaluronic acid, and glycosaminoglycans.[22] This complex matrix also plays a role in migration and support activity of fibroblasts, including sending signals for angiogenesis, granulation tissue regeneration, and epithelialization.[23]

A previous study on the effect of the *Chana striata* extract on the fibroblast count of oral mucosa wound healing revealed that the fibroblast count reaches its peak on day 7. In that study, the fibroblast count increased on day 3 and reached the highest at day 7.[21] Another previous study that investigated the effect

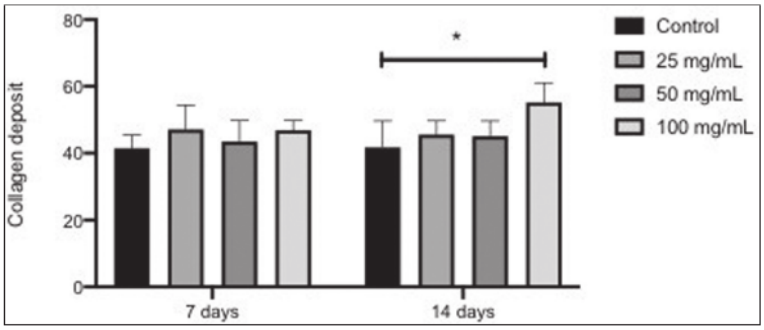

Figure 4: Effect of CNE on oral mucosal burns on days 7 and 14 based on collagen deposits. *$P < 0.05$ between groups. Data are means ± SD, $n = 5$

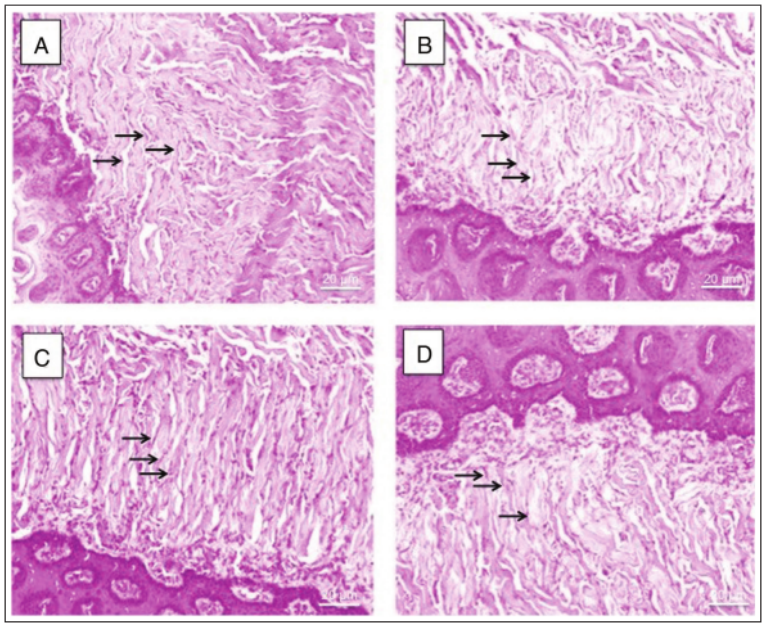

Figure 5: Representative images of the histological view of fibroblasts (black arrow) on the tongue wound of rat tissues on day 7, which are stained with HE. A. Control (untreated). B. 25 mg/mL of CNE. C. 50 mg/mL of CNE. D. 100 mg/mL of CNE. Original magnification 40×

of *Musa acuminata* on the fibroblast count of the oral mucosa of wistar rat revealed that on day 7, the fibroblast count increases in comparison to the negative control and positive control (Aloe vera).[24] In line with this study, the fibroblast count also increased on day 7.

Fibroblasts synthesize a number of growth factors, including fibroblast growth factor-2, platelet-derived growth factor, connective tissue growth factor, insulin-like growth factor-1, and transforming growth factor-β1 (TGF-β1), which are stored in the wound matrix and subsequently stimulate the secretion of the matrix and proliferation of fibroblasts itself.[25] The differentiation of fibroblasts to myofibroblasts is a key event in the wound healing of connective tissues. Expression of the actin isoform alpha-smooth muscle actin (α-SMA) by myofibroblasts is the main characteristic of this cell, rendering the capability to increase contractile forces and reinforce cell matrix adhesion.[26] The contractile forces aim to bring the edges of an open wound together; therefore, it supports wound closure. However, exaggerated activities of fibroblasts cause scar formation and tissue fibrosis.[22]

In this study, the fibroblast count of the oral mucosal burn and tongue wound experiments decreased on day

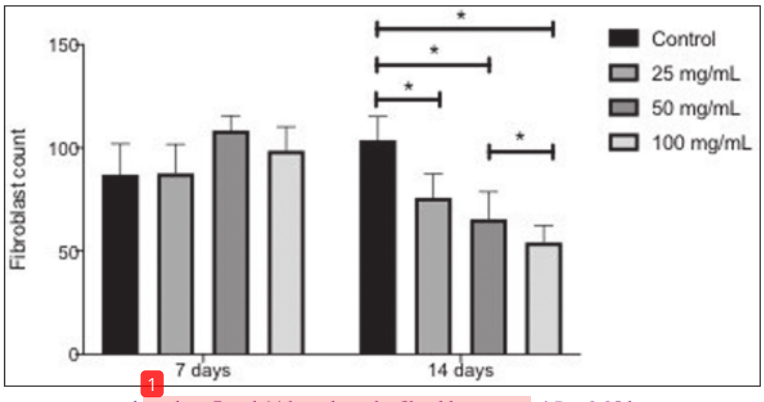

Figure 6: Effect of CNE on the tongue wound on days 7 and 14 based on the fibroblast count. *$P < 0.05$ between groups. Data are means ± SD, $n = 5$

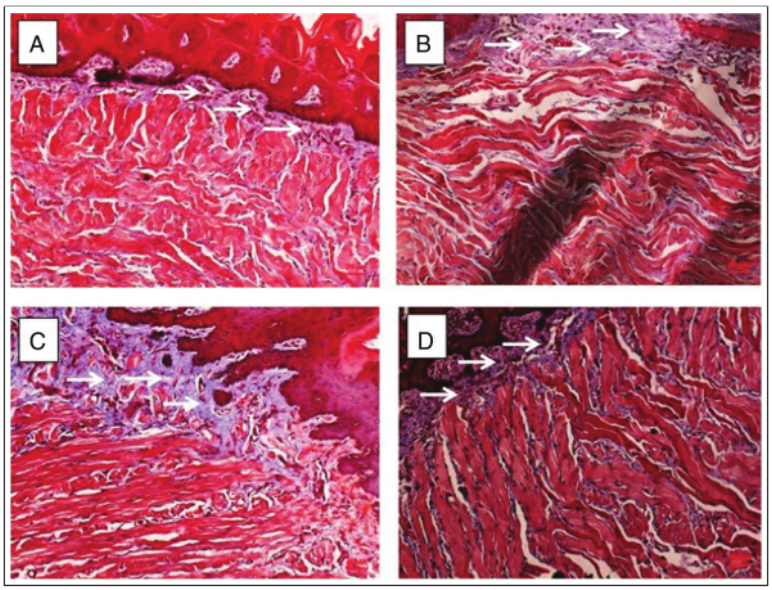

Figure 7: Representative images of the histological view of collagen deposits (white arrow) on the tongue wound of rat tissues on day 14, stained with Masson's trichrome. A. Control (untreated). B. 25 mg/mL of CNE. C. 50 mg/mL of CNE. D. 100 mg/mL of CNE. Original magnification 40×

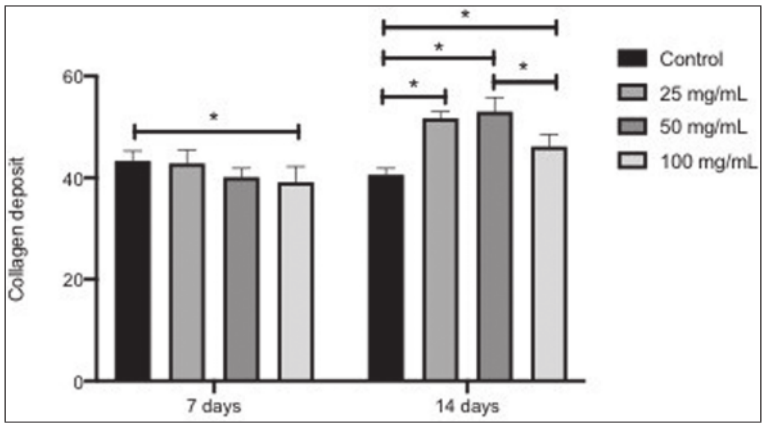

Figure 8: Effect of CNE on tongue wound on days 7 and 14 based on collagen deposits. *$P < 0.05$ between groups. Data are means ± SD, $n = 5$

14. In all treatment groups, the fibroblast count on day 14 was less than that in the control group, probably because the fibroblasts in the treatment group have already synthesized a sufficient amount of collagen, followed by the apoptotic mechanism, whereas in the control group, the synthesized collagen was not sufficient; hence, the fibroblast count was still high at the wound site. Collagen deposits on day 14 in all treatment groups were greater than those in the control group, even though no significance was observed [Figures 6 and 8]. On day 14, the fibroblast count decreased as it was the late stage of proliferation.[22] After the wound area was filled with collagen, the endothelial cell proliferation and fibroblast count decreased, but fibroblasts became more progressive in synthesizing collagen, thereby increasing the number of ECM.[27]

For the mucosal burn experiment, the synthesized collagen on day 7 for the treatment groups was greater than that for the control group. The highest amount of synthesized collagen was observed for the 25 mg/mL treatment group, and the lowest amount of synthesized collagen was observed for the 50 mg/mL treatment group. This result indicated that on day 7, the CNE is more active to induce fibroblasts for collagen synthesis. However, on day 7 for the tongue experiments, the opposite result was observed, and the collagen deposits for the treatment groups were less than those observed for the control group, even though it did not exhibit significance. During the proliferation stage, macrophages stimulate the fibroblast activity via the secretion of TGF-β1.[25] The TGF-β1 was also capable of stimulating fibroblasts to increase the α-SMA level.[28]

Similar to a previous study, acai berry water extract exhibited an increased number of collagen deposits on wound oral mucosa on day 6. In that study, a decreased number of mast cells was noted on day 6, indicating that wound healing is improved via a decrease in inflammation.[29,30] Although the number of collagen deposits on day 7 was less than that observed for the control group in the tongue experiments, on day 14, the opposite result was observed.

Based on the results, all of the concentrations of the CNE revealed the same activity in terms of the fibroblast count and collagen deposits; therefore, it is crucial to conduct further research to investigate the appropriate dosage for wound healing. Phytochemical assay of ethanol extracts of *C. nutans* revealed that this plant contains flavonoids, saponins, alkaloids, triterpenoids, phenolic acids, tannins, coumarins, and lignins.[31] Flavonoids and saponins exhibit extensive biological activities, including antioxidant, anti-inflammatory, and antimicrobial effects. Antioxidant activity can inhibit free radicals, and lipid peroxidation is known to be reduced by not only preventing or slowing down the emergence of cell necrosis but also by increasing vascularity.[32] Antioxidant property accelerates the healing process via the control of oxidative stress.[33] The compounds that can inhibit lipid peroxidation are thought to be able to increase the viability of collagen fibrils via the increase in the strength of collagen fibers as well as the rate of epithelialization.[32]

Flavonoids can inhibit MMPs and increase the rate and amount of collagen synthesized by fibroblasts needed for the formation of a new wound matrix; hence, the wound healing process is accelerated. Anti-inflammatory and antimicrobial activities in these compounds are related to the decreased number of microbes in the wound site and shortening of the inflammatory stage; hence, re-epithelialization is accelerated, and tissue

reorganization is better.[34] Saponins can stimulate the synthesis of fibronectin by fibroblasts and change the expression of TGF-β receptors.[35]

Results from a recent study are in agreement with those of previous studies that investigated chloroform extracts and isolated purpurin-18 phytyl ester from *C. nutans* using an *in-vitro* wound healing assay. The result revealed that the crude chloroform extract and isolated compound can accelerate the migration of human gingival fibroblasts.[12] The difference in the methods used in their study (i.e., solvent extraction) and that used herein may affect the result. A previous study has used chloroform and Soxhlet extraction methods,[12] whereas in this study, ethanol and maceration extraction were employed. Therefore, further research is required to determine the best solvent and extraction method for the CNE for wound healing.

Conclusion

In conclusion, CNE can increase the fibroblast count on day 7 and decrease that on day 14 in mucosal burns and tongue wound experiments. This extract also increases the number of collagen deposits on day 14 for both experiments in the wound healing process of Sprague Dawley rats.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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