JTL 11 (2) December 2022
Page: 52-59

Contents list available at Jurnal Teknologi Laboratorium

JURNAL TEKNOLOGI LABORATORIUM

Journal Homepage: www.teknolabjournal.com
ISSN 2580-0191(Online) I ISSN 2338 – 5634(Print)

Original Research

OPEN ACCESS

The wound healing effect of Morinda citrifolia leaf extract and biomolecular analysis on inflammation and proliferation stages in Wistar rats

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Abstract: Noni leaves (Morinda citrifolia L.) are empirically used to heal wounds traditionally. The active ingredients of the leaves have benefits as antibacterial, anti-inflammatory, antioxidant, antiviral etc. This research aims to evaluate the wound healing effect of noni's leaf extract (*Morinda citrifolia L.*) based on phytochemical analysis, the number of fibroblast cells, wound diameter, healing time, TNF-α and IL-1 at inflammation stage, PDGF and TGF-β at proliferation stage. This study is a true experimental with a post-test-only control group design. We divided 25 male Wistar rats into five groups; positive control (with povidone-iodine), negative control (with no treatment), treated by NLEE 20%, 25%, and 30%. NLEE has good physical characteristics based on the value of ethanol soluble extract, water-soluble extract, water content, ash content, and acid insoluble ash content. GCMS analysis showed bioactive compounds such as N-ethyl hydrazine carbothioamide (31.57%), 2-furancarboxaldehyde 5-hydroxymethyl (15,64%), 2E-3,7,11,15-tetramethyl-2-hexadecene-1-of (7.08%), 3,5-dihydroxy-6-methyl-2,3-dihydro-4H-pyran-4-one (4.29%), D:C-friedooleana-7,9(11)-dien-3-ol,3 beta (4.04%), while the rest are considered as the decanoic acids methyl ester, piperazine (3%) and vitamin E (1%). We observed the slight effects of NLEE 20%, 25%, and 30% on the number of fibroblasts, wound diameter, healing time, TNF-α, IL-1, PDGF, and TGF-β. The optimum concentration of NLEE at any treatment was 20%. However, there were no significant differences between groups based on a two-way ANOVA analysis. This research implies a potential utilization of noni leaves as the alternative for wound healing treatments.

Keywords: Morinda citrifolia; Wound healing; Inflammation; Proliferation

INTRODUCTION

Physical and chemical trauma can cause injury [1] and disruption of skin integrity [2,3]. The side effect of commercial medicine is one of the reasons to continue the research on herbal therapy that effective and safe for wound healing application [4]. Hence, we studied a Noni leaf extract (*Morinda citrifolia L*) which was previously reported for its antibacterial, antiinflammation, antioxidants, antivirus, etc [5,6]. The potential bioactivities were supposed due to the presence of secondary metabolites; alkaloids for antibacterial, flavonoids for antiinflammation, saponins for antiseptic, tannins, and triterpenoids for antioxidants [7].

Previous studies reported that hydro alcohol extract [8], ethanol extract [9], hexane fraction [10], and cream of ethanol extract [11] have positive effects on wound healing, even though no significant effect on the healing of rat oral mucosal ulceration. A significant difference in fibroblast cells of Wistar rats was reported

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DOI: 10.29238/teknolabjournal.v11i2.369
Received 21 July 2022; Received in revised form 07 August 2022; Accepted 06 December 2022

between the cream's extract of 10% and control as well as paste's extract could reduce the inflammation of the carrageenan-induced rat paw edema [12].

The comprehensive research covering phytochemical analysis and biomarkers investigation on inflammation and proliferation stage on wound healing treated with noni's leaves ethanol extract (NLEE) is not reported yet. In this study, we report the phytochemical analysis by using GCMS then followed by wound healing evaluation based on the number of fibroblasts, wound diameter, healing time, TNF-α and IL-1 at inflammation stage, PDGF and TGF-β at proliferation stage. Data obtained were evaluated by SPSS with analysis of variance (ANOVA).

MATERIAL AND METHOD

The noni's leaves were obtained from the university's garden and then washed and dried. Noni leaves were determined in Herbarium Universitas Sumatera Utara. Simplicia powder was prepared by using a mixer, then macerated with ethanol 96% for 1 day, 3 times. After rotary evaporation, noni's leaves ethanol extracts (NLEE) were varied by 20, 25, 30 (% w/w) concentrations. Laboratory tools used were rotary evaporator (B-One), separating funnel (Schott Duran), and Waterbath (memmert). Solvents and reagents used were aquadest, ethanol 96%, chloralhydrate, ethanol 96%, ethyl acetate, methanol, chloroform, n-hexane, FeCl, bourchardat reagent, Mayer's reagent, NaOH, H_2SO_4, aquadest, and dragendor's reagent.

This study is a true experimental with a post-test-only control group design. About 25 male Wistar rats (2-3 months,150-200 g) were acclimatized for one week and selected according to the completely randomized design method. The animal experiment procedure has been approved by Ethics Committee (KEPK UNPRI) No. 029/KEPK/UNPRI/X/2021.

The animal groups are:

K- : Negative control, with no treatment.
K+ : Positive control, with Povidone Iodine.
PI : treated with NLEE 20%
PII : treated with NLEE 25%
PIII : treated with NLEE 30%

The animals hair were shaved and then anesthetized with lidocaine. The incision was made on the back using a sterile scalpel 2 cm long. The wound was smeared with NLEE 2 times per day at 08.00 am and 05.00 pm from day 1st to day 14th. Wound observations were carried out visually every day by measuring the diameter of the wound, and calculating the average reduction in wound diameter and healing days. Wounds are considered healed if the diameter of the wound is zero. Wounds closure formula was referred to Guo et.al., 2020. Each group was evaluated by the number of fibroblasts at 400 magnifications, and immunohistochemistry expression of TNFα, IL-1, TGFβ , and PDGF on days 3rd, 7th, and 14th.

Statistical analysis has been employed to determine the normality of data obtained by using the *Kolmogorov-Smirnov* test ($p > 0.05$) and the homogeneity by *Levene* methods. Then two-way *ANOVA* was applied ($p < 0.05$), followed by the *Post Hoc Tukey HSD* test to determine the significant differences between groups.

RESULTS AND DISCUSSION

NLEE contains 44.72% ethanol soluble extract, 15.32% water soluble extract, 8.64% water content, 8.56% ash content, and 1.26% acid insoluble ash content. A qualitative phytochemical method showed that NLEE has a positive reaction with all reagents indicating that NLEE contains alkaloids, flavonoids, glycosidic, saponins, tannins, triterpenoids/steroids. Quantitatively, NLEE has 10.88 mgQE/g of flavonoid content.

GCMS analysis showed that NLEE mostly contained n-ethyl hydrazine carbothioamide (31.57%), 2-furancarboxaldehyde 5-hydroxymethyl (15,64%), 2e-3,7,11,15-tetramethyl-2-hexadecene-1-ol (7.08%), 3,5-dihydroxy-6-methyl-2,3-dihydro-4H-pyran-4-one (4.29%), D:C-friedooleana-7,9(11)-dien-3-ol,3beta (4.04%). The rest compounds are decanoic acids methyl ester, piperazine (3%), and vitamin E (1%). The specific numbers of the GCMS spectrum as shown in Figure 1.

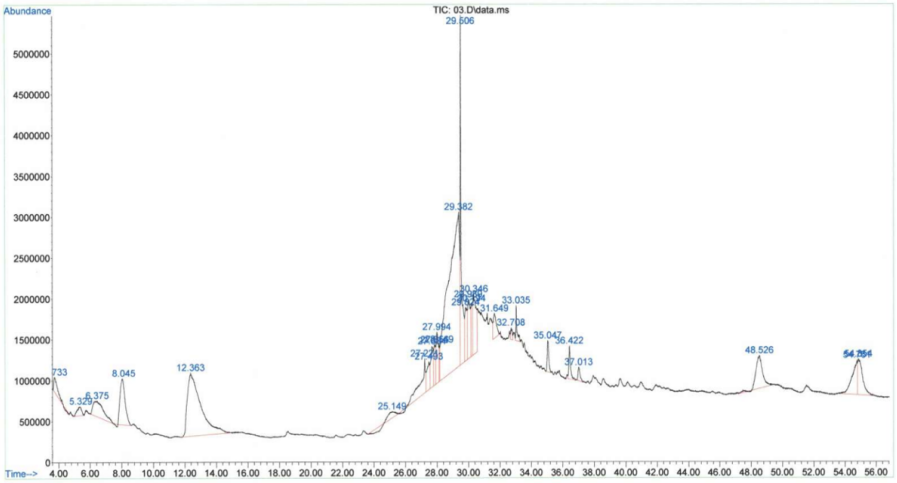

Fig 1. GCMS spectra of NLEE

Literature studies showed that hexanoic acid (3.02%) and tetradecanoic acid (1.36%) were saturated fatty acids with potential as antioxidants anticancer and antihistamines [13]. The 2-furancarboxaldehyde 5-hydroxymethyl (15,64%) and 3,5-dihydroxy-6-methyl-2,3-dihydro-4H-pyran-4-one (4.29%) were phenolic compounds that reported as antimicrobial, anti-inflammatory and antiproliferative [14]. Vitamin E is the popular antioxidant as well as the piperazine known as anthelmintic.

Kolmogorov-Smirnov test showed that the number of fibroblasts, wound diameter, healing time, TNFα, IL-1, TGFβ, and PDGF data are normal ($p > 0.05$). *Levene* test showed that the regression model meets the assumption of homogeneity ($p > 0.05$).

The number of fibroblast cells with hematoxylin staining produced on a variety of NLEE is shown in Figure 2.

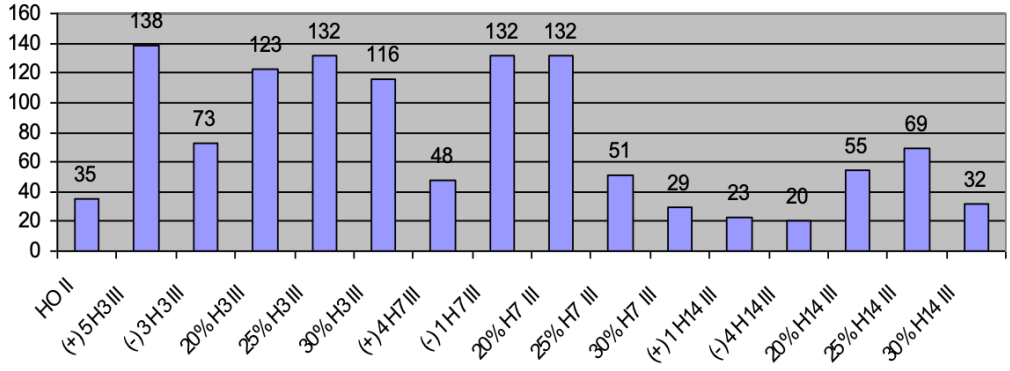

Fig.2 Effect of NLEE on the number of fibroblast cells on day 3rd,7th, and 14th between positive control (+), negative control (-), NLEE 25%, 25%, and 30%.

Figure 2 indicates that the variety of NLEE had no significant effect on the number of fibroblast cells. Even though there were changes in the number of fibroblasts but no significancies showed by two-way ANOVA (sig 0.885, α 5%).

This result is in line with a report that showed no significant effect of noni's extract against the number of fibroblast cells[6]. In their report, the number of fibroblasts in the control group was higher than in all treatment groups, although it was not statistically significant. This indicates that the proliferation of fibroblast cells in all treatment groups has decreased earlier than in the control group.

This phenomena could be explained that occurrence of fibrosis was subjected to the inflammatory response classified as acute and chronic inflammation, which differs from their vascular reactions, cells involved, and duration [6]. Another report also showed that a remodelling phase of wound healing, fibroblast cell proliferation decreases as collagen fibres are synthesized [12].
The wound diameter and healing time on a variety of NLEE are shown in Fig 3.

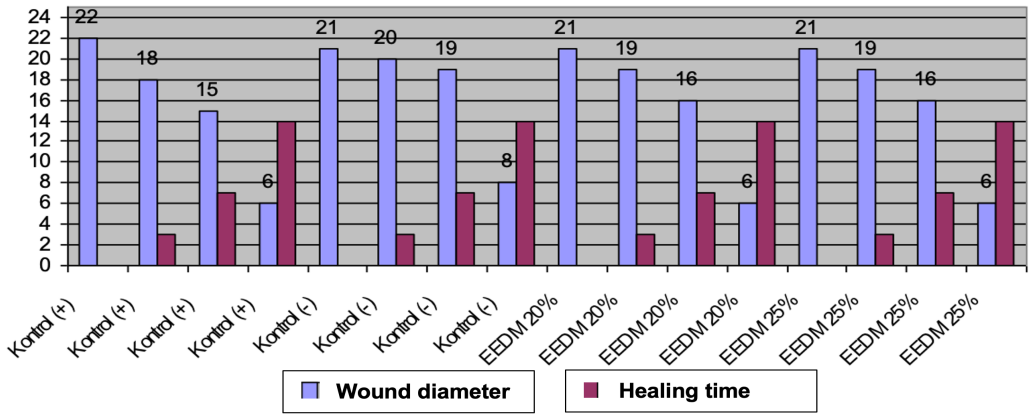

Fig.3 Effect of NLEE on the wound diameter and healing time on the day 3rd,7th, and 14th between positive control (+), negative control (-), NLEE 25%, 25%, and 30%

Figure 3 indicates that the variety of NLEE had no significant effect on the wound diameter and healing time. Even though there were but no significancies showed by two-way ANOVA (sig 0.997 and 1.000 with α 5% for wound diameter and healing time respectively). Our result was the same as the report by [15]. This phenomenon explained that wound healing in the skin is a complex process involving blood clotting, inflammation, new tissue formation, and tissue remodelling [16].

The effects of NLEE on TNFα and IL-1 were shown in Figures 4 and 5 respectively.

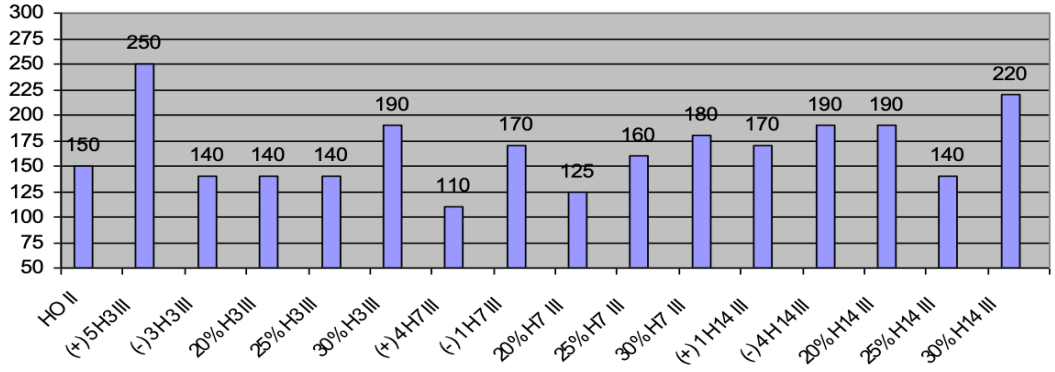

Fig.4 Effect of NLEE on the TNFα on the day 3rd,7th, and 14th between positive control (+), negative control (-), NLEE 25%, 25%, and 30%.

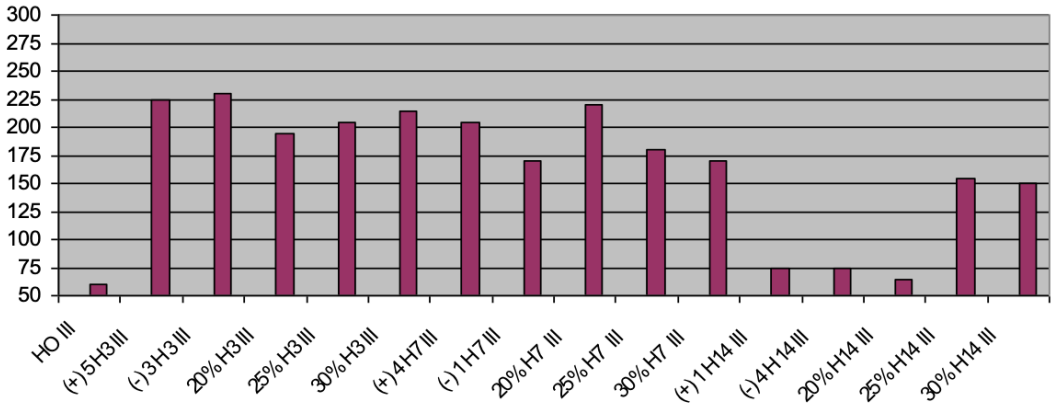

Fig.5 Effect of NLEE on the IL-1 on the day 3rd,7th, and 14th between positive control (+), negative control (-), NLEE 25%, 25%, and 30%.

Figure 4 showed that the variety of NLEE had no significant effect on the TNFα by two-way ANOVA (sig 0.541 with α 5%) nor the IL-1 (sig 0.949 with α 5%) as shown in Figure 5.

A previous report showed that the variety of noni's leaf extract did not have a significant effect on the expression of TNF-a and IL-1 [17]. In their report, noni's extract showed maximal inhibition of IL-1β production (79%) at 750 g/ml, which was not statistically different from the maximal inhibition produced by dexamethasone (97% at 3.92 g/ml), indomethacin (70% at 3.58 g/ml), and rutin (90% at 48.84 g/ml). A comparison of IC_{50} values revealed that the inhibitory potential of IL-1β production from the extract (294 g/ml) was much lower than that of rutin (30.2 g/ml), dexamethasone (0.93 g/ml), and indomethacin (1.50 g/ml). This can be explained by that TNF-α plays a key role in the induction and perpetuation of inflammation and up- regulation of other pro-inflammatory cytokines and endothelial adhesion molecules[18]. The production of TNF-α increases the release of IL-1β [19,20].

The effects of NLEE on TGFβ and PDGF were shown in Figures 6 and 7 respectively.

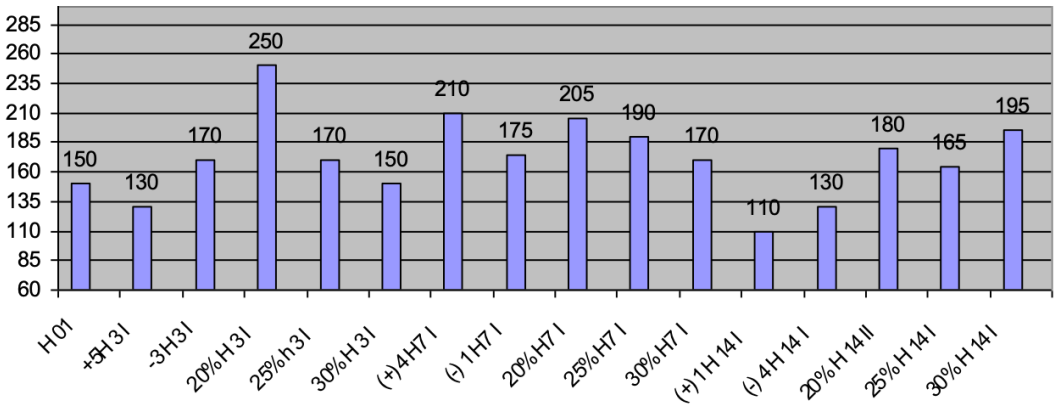

Fig.6 Effect of NLEE on the TGFβ on the day 3rd,7th, and 14th between positive control (+), negative control (-), NLEE 25%, 25%, and 30%.

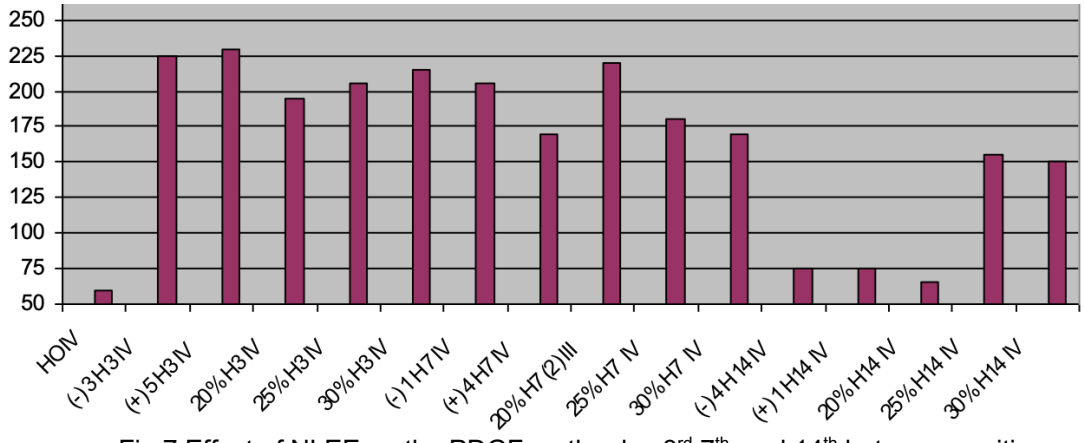

Fig.7 Effect of NLEE on the PDGF on the day 3rd,7th, and 14th between positive control (+), negative control (-), NLEE 25%, 25%, and 30%.

Figure 6 showed that the variety of NLEE had no significant effect on the TGFβ by two-way ANOVA (sig 0.510 with α 5%) nor the PDGF (sig 0.997 with α 5%) as shown in Figure 7. Based on data shown in Figures 2-7, the descriptive analysis showed that the optimum condition was at NLEE 20% with a mean value of 1.9625 (sig. 0.310 α 5%). During the inflammation stage in the first three-days injury, the wound healing effect is related to the degradation of cytokine activity released by macrophages; TNF-α, IL-1, PDGF dan TGF-β [21]. Afterward, during the proliferation stage, the PDGF and TGF-β will be secreted after injury immediately. PDGF can increase fibroblast proliferation and then produce an extracellular matrix whereas the PDGF-BB gene-modified human Dental Pulp Stem Cells (hDPSCs) can continuously secrete the PDGF-BB protein and that the overexpression of PDGF-BB can significantly enhance hDPSC proliferation[22]. TGF-β also plays an important role to increase fibroblasts, and cell chemotaxis, and modulates collagen and collagenase expression, which in turn produces matrix-producing cells for the rapid deposition of the new connective tissue[23]. In adult mammals, high levels of TGFβ1 and TGFβ2, and low levels of TGFβ3 facilitate scar-forming healing, while in fetal mammals, high levels of TGFβ3 and low levels of TGFβ1 and TGFβ2 favour scar-free healing[24]. The multifunctional cytokine plays a central role in tissue repair. Initially after wounding, TGF-β is secreted by platelets, and serves as a chemoattractant for parenchymal cells and leukocytes, which in turn enhance production of TGF-β. This mechanism leads to restoration of tissue architecture in normal wound repair, although overproduction may lead to fibrosis [25].

CONCLUSION

Simplicia of noni's leaf extract has several bioactive compounds based on GCMS data. Quantitatively, NLEE has 10.88 mgQE/g of flavonoid content. Some phenolic compounds and saturated fatty acid methyl ester, piperazine, and vitamin E are the bioactive compounds that play a role in the wound healing effect in Wistar rats. We observed the slight effects of NLEE 20%, 25%, and 30% on the number of fibroblasts, wound diameter, healing time, TNF-α, IL-1, PDGF, and TGF-β. The optimum concentration of NLEE at any treatment was 20%. However, there were no significant differences between groups based on a two-way ANOVA analysis. These results suggest a diverse variety of concentrations to capture a better understanding of the wound healing potential of NLEE treatments in Wistar rats.

AUTHORS' CONTRIBUTIONS

All authors contributed equally to this work.

ACKNOWLEDGEMENT
Author thanks to Pharmacy Laboratory, Universitas Sumatera Utara, for their facilities and materials during this research.

FUNDING INFORMATION
This research did not receive any specific grant from funding agencies

DATA AVAILABILITY STATEMENT
The utilized data to contribute to this investigation are available from the corresponding author on reasonable request.

DISCLOSURE STATEMENT
The views and opinions expressed in this article are those of the authors and do not necessarily reflect the official policy or position of any affiliated agency of the authors. The data is the result of the author's research and has never been published in other journals.

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