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# Efficacy of Transdermal Delivery Nano Ethosomal Gel from Ashitaba Leaves on In-vivo Burn Wound Healing in Albino Rats

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## Abstract

This study aimed to evaluate the in-vivo burn wound healing process in albino rats treated using transdermal delivery nano ethosomal gel from ashitaba leaves. Ethosomal vesicles were formulated using soy lecithin, cholesterol, ethanol, water, and ashitaba leaf nanoparticles using the cold method. A total of 25 male rats were randomly divided into 5 groups, i.e., (C-) treated without nanoparticle extract, (C+) treated using 1% Silver Sulfadiazine®, treatment group treated nano ethosomal gel from ashitaba leaves with the respective doses were (T1) 1%, (T2) 2,5%, and (T3) 5%. Therapy was initiated on day 1 or after being induced by a burn wound for 14 days, twice a day. On the 15th day, a termination was carried out to take skin tissue from burn scars, then histopathology preparations were made with routine staining. Microscopic observations with an optical microscope on collagen, polymorphonuclear cell infiltration, angiogenesis, and re-epithelialization. The T3 group that was treated using 5% nano ethosomal gel showed the best burn wound healing, this may be caused by compounds in ashitaba leaf nanoparticles which have antioxidant, anti-inflammatory, and antibacterial effects, thus the use of transdermal delivery therapy of 5% ashitaba leaf nano ethosomal gel was effective for wound treatment burn on rat skin.

Keywords: ashitaba leaves, burns, ethosomal gel, nanoparticles, wound healing

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## INTRODUCTION

Burns are a serious public health problem characterized by injury to the skin or tissue damage. Burns can be caused by contact with heat, radiation, radioactivity, electricity, friction, or chemicals (Bhatia *et al*., 2014). The World Health Organization (2020) reported that 180.000 deaths occur every year throughout the world due to burns. The majority of burn injuries occur in low and middle-income countries. The incidence of burn deaths in children is more than 7 times higher than in high-income countries. In Indonesia, burns cause around 195.000 deaths each year. The Indonesian National Burn Injury Referral Center records that there are more than 130 patients referred each year from various countries. Mortality analysis of adult burn patients at Cipto Mangunkusumo Hospital (2009–2010) and Dr. Soetomo (2007–2011) reached 34% and 14,5% (Wardhana *et al*., 2017).

Burns have the potential to destroy skin and other tissues such as blood vessels, nerves, tendons, and bones (Yuniarti and Lukiswanto, 2017). Extensive burns cause the patient's body to be unable to tolerate the condition resulting in death and require special treatment (Berrak and Yegen., 2004). Long-term care for burn patients typically involves reconstructive surgery, hospitalization, and frequent visits to healthcare facilities which also places a socioeconomic burden on victims and caregivers (Smolle *et al*., 2017).

Healing burns can be done using natural ingredients that have the potential to act as anti-inflammatory, antioxidant, and antibacterial such

as the ashitaba plant (*Angelica keiskei*). This plant is used as a traditional medicine originating from Japan. The ashitaba plant also has potential as a medicine because its yellow sap contains the substance chalcone. Chalcone has been proven to have chemopreventive, antibacterial, antioxidant, and anti-inflammatory properties (Caesar and Cech, 2016). Antioxidants from ashitaba leaves can trigger collagen production and increase Vascular Endothelial Growth Factor (VEGF). Anti-inflammatory activity occurs by reducing inflammatory symptoms such as pain, redness, and swelling, while antibacterial activity occurs by inhibiting bacterial growth. The active substances contained in chalcone are useful in increasing the body's defenses to fight against infectious diseases (Venugopal, 2015; Solikhah *et al*., 2022).

The efficiency of transdermal delivery therapy for burn wounds can be provided in the form of nano ethosomal gel which is a new vesicular carrier to increase delivery through the skin. The size of ethosomal nanovesicles can be modulated from microns to nano micros. Ethosomal is an innovative nanovesicle that contains drugs in a lipid, ethanol, and water matrix or is defined as a modified form of liposomes with a high ethanol content (Shelke and Kulkarni, 2018). This preparation can penetrate the skin and increase the delivery of compounds to both the dermis and systemic skin strata. Ethanol contained in ethosomal fluid can penetrate the lipid bilayer found in the stratum corneum (Venugopal, 2015).

Nano ethosomal gel can increase the bioavailability of drugs so that they can penetrate the spaces between cells even in small organs. Another advantage of nano ethosomal gel is that it can increase the target specificity of the drug so that it is more precisely targeted (Shelke *et al*., 2015). Appropriate treatment of burns requires attention, especially on the effectiveness of therapy because the incidence rate is still quite high. The aim of this study was to evaluate the effectiveness of transdermal delivery of ashitaba leaf nano ethosomal gel on the in-vivo burn wound healing process in rats.

## MATERIALS AND METHODS

### Ethical Approval

All study procedures were evaluated and approved by the Ethics Committee of the Faculty of Veterinary Medicine, Universitas Airlangga, Surabaya, Indonesia on May 16 2019 with certificate No.2.KE.092.05.2019.

### Preparation of Ashitaba Leaf Extract

Ashitaba plants were obtained from Trawas, Mojokerto, East Java, Indonesia, and received a certificate from Overseas Merchandise Inspection CoLTD (OMIC). Ashitaba leaves that have been picked, washed, and chopped are then air-dried indoors to dry for 7 days. The dried ashitaba leaves were then ground until smooth, and then macerated by soaking in a 96% ethanol solution with a ratio of 1:10 for 5 days. The extract was obtained from maceration which is then evaporated using an evaporator at a temperature of 60°C (Kusuma *et al*., 2018).

### Preparation of Nano Ethosomal Gel

Nanoparticle production of ashitaba leaf was performed using a ratio of 1:1:6 or 10 ml of 5% ashitaba leaf extract, 10 ml of 0,1% NaTPP, and 60 ml of 0,2% chitosan. The materials were mixed and sonicated with a sonicator machine for 60 minutes at a frequency of 20 kHz, then finally dried using freeze drying.

Nano ethosomal gel production from ashitaba leaf nanoparticles using the cold method. Accurately weigh the soya lecithin and dissolve it in water as needed. Ashitaba leaf nanoparticles and cholesterol were weighed and dissolved in ethanol as needed, then transferred into the soya lecithin solution and stirred until a homogeneous solution was obtained. Propylene glycol was added and mixed thoroughly. This mixed solution is then sonicated or extruded to reduce the size of the vesicles for 10 minutes. The results of this process are then packaged in glass bottles and stored in the refrigerator for further use (Patel, 2007; Sheer and Chauhan, 2011; Panchagnula *et al*., 2004). The nano ethosomal formulation of ashitaba leaves can be seen in Table 1.

Nano ethosomal gel of ashitaba leaf mixed from dissolving methylparaben and propylparaben in 50 ml of water and the required amount of carbopol was added until it was homogeneously dispersed. Propylene glycol and EDTA were added to the above mixture and triethanolamine dissolved in 25 ml of water was added to maintain the desired pH, then the prepared ashitaba leaf nano ethosomal was added and dispersed well to obtain ashitaba leaf nano ethosomal gel (Jain, 2001; Barry, 2001). The formulation of nano ethosomal gel in ashitaba leaves can be seen in Table 2 and the results of the phytochemical test of nano ethosomal gel in ashitaba leaves can be seen in Table 3.

**Induction of Burn Wounds in Rats**

Rats were acclimatized in the laboratory for 1 week. On the 8th day, a burn wound was made on the skin of the rat's right gluteus area which had been shaved with an area of 2x2 cm. Before shaving, anesthesia was carried out using a dose of Ketamine 100 mg/kg and Xylazine 5 mg/kg. Induction of burns using a thermostat modified with a round stainless steel plate with a diameter of 1 cm, pressed firmly against the surface of the shaved skin (Porumb *et al*., 2017), for 5 seconds at a temperature of 85°C with an average tolerance of 85 ± 5°C (Abdeldjelil *et al*., 2017).

**Treatment of Burn Wounds in Rats**

This study was an experimental type of study using 25 male rats, weighing 150–200 g, randomized into five treatment groups with 5 replications each, i.e., (C-) treated burns and gel without nanoparticle extract, (C+) treated burn wounds and were given 1% Silver Sulfadiazine® gel, the treatment group was treated burn wounds and ashitaba leaf nano ethosomal gel treatment with respective doses, i.e., (T1) 1%, (T2) 2.5%, and (T3) 5%. Treatment therapy was initiated on day 1 or after induction of burns for 14 days, twice a day (Akbari *et al*., 2015).

**Routine Staining**

Rats were euthanized first using a combination of ketamine 300 mg/Kg BW and Xylazine 30 mg/Kg BW intraperitoneally. After that, skin samples from burn scars were taken at the end of the experiment by excision of 2 x 2 cm and fixed in 10% buffered formalin for histopathological preparations. Specimens were stained using hematoxylin eosin. Collagen scoring, number of polymorphonuclear cells (PMN), angiogenesis, and re-epithelialization were evaluated using microscope CX41 Olympus Japan with 400x magnification (Table 4) (Shakya *et al*., 2016; Nasiri *et al*., 2015).

**Data Analysis**

The data obtained were analyzed using the SPSS 23.0 software program (IBM, USA) to analyze the comparison between the treatment and control groups. The normality test was carried out using Shapiro-Wilk. Non-parametric tests were carried out using the Kruskal-Wallis test followed by the Mann-Whitney test with a significance level of $p < 0,05$.

## RESULTS AND DISCUSSION

The results of collagen assessment and the number of PMN in this study showed similar. C- compared with T3 group showed a significant difference ($p < 0,05$), but did not show a significant difference ($p > 0,05$) compared with C+, T1, and T2. An evaluation of angiogenesis between C-, C+, T1, and T2 did not show a significant difference ($p > 0,05$), but had a significant difference ($p < 0,05$) with T3 group. The re-epithelialization scores at C- and T3 showed significant differences ($p < 0,05$) with C+, T1, and T2. The highest re-epithelialization score was seen at T3 and had a significant difference ($p < 0,05$) with other treatment groups (Table 5).

In general, inflammatory process, re-epithelialization, and angiogenesis of the treatment groups were better than C- group. The best results from the wound healing process were observed in T3 with its characteristics showing high collagen density with good arrangement accompanied by complete and mature epithelium, low inflammation, and angiogenesis. The histopathological figure of skin with burns wound on the 15th day was reported in Figure 1.

**Table 1.** Ashitaba leaf nano ethosomal formulation

| Ingredients | Quantity (%) |
|---|---|
| Ashitaba leaf nanoparticles | 1 |
| Soya lecithin | 4 |
| Cholesterol | 0,5 |
| Ethanol | 45 |
| Propylene glycol | 20 |
| Water | Quantum satis |

**Table 2.** Nano ethosomal gel formulation

| Ingredients | Ethosomal gel (%) | Gel (%) |
|---|---|---|
| Nanoethosomes | 1 | - |
| Ashitaba leaf nanoparticles | - | 1 |
| Carbopol | 2 | 2 |
| Propylene glycol | 15 | 15 |
| Sodium edentate | 0,01 | 0,01 |
| Propylparaben | 0,001 | 0,001 |
| Methylparaben | 0,05 | 0,05 |
| Triethanolamine | Quantum satis | Quantum satis |
| Water | Quantum satis | Quantum satis |

**Table 3.** Phytochemical evaluation for the nano ethosomal gel content of ashitaba leaves

| Phytochemical test | TLC scanning |
|---|---|
| Flavonoids | + (positive) |
| Polyvenol | + (positive) |

**Table 4.** Histological observation scoring

| Parameters | Scoring | | | |
|---|---|---|---|---|
| | None | Mild | Moderate | Severe |
| Collagen | 0 | 1 | 2 | 3 |
| Number of PMN | 0 | 1 | 2 | 3 |
| Angiogenesis | 0 | 1 ( ≤ 5 vein) | 2 (6–10 vein) | 3 ( ≥ 11 vein) |
| Re-epithelialization | 0 | 1 (partially) | 2 (complete but with immature epithelium) | 3 (complete with mature epithelium) |

**Table 5.** Skin histopathology scores in the treatment group

| Groups | Mean ± SD | | | |
|---|---|---|---|---|
| | Collagen | PMN | Angiogenesis | Re-epithelialization |
| C- | $1,80^{a} \pm 0,45$ | $3,00^{a} \pm 0,00$ | $3,00^{a} \pm 0,00$ | $0,20^{a} \pm 0,45$ |
| C+ | $2,60^{ab} \pm 0,55$ | $2,20^{ab} \pm 1,10$ | $2,20^{a} \pm 0,84$ | $1,40^{b} \pm 1,14$ |
| T1 | $2,60^{ab} \pm 0,55$ | $2,20^{ab} \pm 0,45$ | $2,40^{a} \pm 0,89$ | $1,20^{b} \pm 0,84$ |
| T2 | $2,60^{ab} \pm 0,55$ | $2,60^{ab} \pm 0,55$ | $2,60^{a} \pm 0,55$ | $1,00^{b} \pm 0,71$ |
| T3 | $3,00^{b} \pm 0,00$ | $1,40^{b} \pm 0,55$ | $1,40^{b} \pm 0,55$ | $2,80^{c} \pm 0,45$ |

$^{abc}$Different superscripts in the same column indicate significant differences ($p < 0,05$).

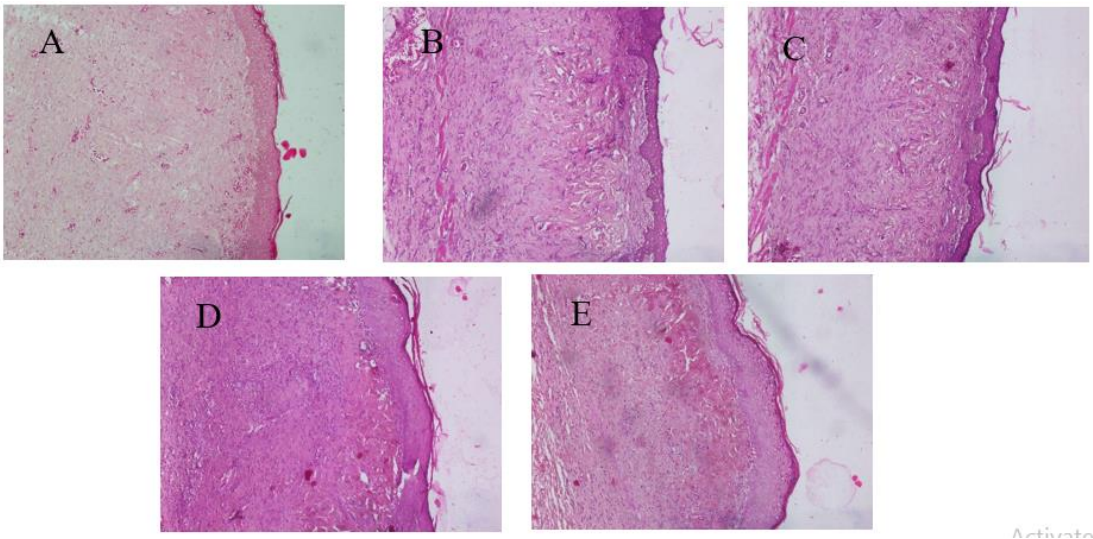


**Figure 1.** The histopathological figure of burn wound in the skin with hematoxylin-eosin staining on the 15th day after treatment. (A) Low collagen and re-epithelialization, high number of PMNs and angiogenesis, (B) Medium collagen, partial re-epithelialization, moderate number of PMNs and high angiogenesis, (C) low collagen, partial immature re-epithelialization moderate number of PMNs, and high angiogenesis, (D) low collagen, complete immature re-epithelialization, low number of PMNs and angiogenesis, (E) high collagen, complete mature re-epithelialization, low number of PMNs and angiogenesis.

The wound healing process generally goes through inflammation, proliferation, and remodeling phases (Yuniarti and Lukiswanto, 2017). This process is not much different from the process of burn healing. Perfect burn wound healing is characterized by complete wound closure in a shorter time span without side effects (Triana *et al*., 2020). However, the healing of burn wounds can be delayed by several factors such as oxidative stress, diabetes mellitus, and microbial infections (Cai *et al*., 2014). In this study, factors that may play a role in inhibiting burn wound healing are oxidative stress and microbial infection. Various studies have proven that infection is the main cause of failure to heal and can even cause death in burn patients, therefore the use of topical medication for burn wounds is thought to require the role of antibacterial, antioxidant, cheap, and does not have dangerous side effects (Ladou and Cohen, 2003).

The C+ group treated using 1% Silver Sulfadiazine® was unable to provide optimal collagen formation results, this was due to the negative effect of Silver Sulfadiazine® which inhibited wound contracture and delayed an incomplete epithelialization (Saeidinia *et al*., 2017). Silver Sulfadiazine® also causes several systemic complications such as neutropenia, erythema multiforme, crystalluria, methemoglobinemia, and delays in the wound healing process (Nasiri *et al*., 2015; Khan and Akhtar, 2022).

The healing process of burn wounds in the T3 group who were treated with 5% ashitaba leaf nano ethosomal gel showed better than the other groups. These results indicate that nano ethosomal gel from ashitaba leaves with a concentration of 5% is the optimum dose that can accelerate the healing process of burn wounds. These findings related to the properties of transdermal delivery of ashitaba leaf nano ethosomal gel in topical burn wound therapy applications, namely showing effective wound healing properties by increasing various phases of the healing process (Rani *et al*., 2022; Rickyawan *et al*., 2022). The potent wound-healing properties of ashitaba leaf nano ethosomal gel may be due to the synergistic activity of the phytochemical constituents. Ashitaba leaf phytoconstituents such as chalcone and coumarin act as antibacterial and anti-inflammatory, then flavonoids and triterpenoids have anti-inflammatory and antioxidant properties with a free radical

scavenger mechanism of action (Al-Anshori *et al*., 2019).

Increased penetration of ashitaba leaf nanoparticles in ethosomal gel occurs by various mechanisms. Nano ethosomal, which is smaller than 300 nm, can pass through the skin membrane to the deepest layers (Samnani *et al*., 2012). The ethanol and propylene glycol content in nano ethosomal increases transdermal penetration and causes drug interactions with stratum corneum lipid molecules (Zhou *et al*., 2018), this will increase the fluidity of the lipid bilayer and lead to an increase in stratum corneum permeability to drugs (Bendas and Tadros 2007). Ashitaba leaf ethosomal nanovesicles formulated into a gel dosage form can release drugs in a controlled manner and increase the residence time of drugs on the skin surface (Iizhar *et al*., 2016; Shelke *et al*., 2015).

Prolonged and excessive inflammatory processes pose a risk of delayed wound healing (Kartikasari *et al*., 2020; Mihardi *et al*., 2022). The inflammation process function is to clean damaged cells, vasodilation, and extravasation of inflammatory cells. The types of leukocytes that play the main role in inflammation are neutrophils and macrophages (Purnama *et al*., 2019). These cells make chemical mediators such as IL-1β and tumor necrosis factor-alpha (TNF-α). High macrophage and neutrophil counts over a longer period of time can cause chonic inflammation which automatically worsens the wound environment. Inflammation can also increase aerobic cell formation and degradation. This mechanism can create the spontaneous formation of reactive oxygen species (ROS) (Röhl *et al*., 2015). High amounts of ROS can cause oxidative stress conditions (Johar *et al*., 2004). Based on these processes, burn wounds that experience a high inflammatory process need to be treated with anti-inflammatory and antioxidant substances to reduce oxidative stress and prevent the possibility of delayed wound healing (Lukiswanto *et al*., 2019).

During the inflammation process, chemical mediators express Cyclooxygenase-2 (COX-2) for the angiogenesis process by encouraging the synthesis of prostaglandins (PGs) which play a role in the induction of Vascular Endothelial Growth Factor (VEGF) (Hamid *et al*., 2018; Puspasari *et al*., 2018). Ashitaba leaf nano ethosomal gel, which has anti-inflammatory properties in this study, can inhibit COX-2 expression by inhibiting the angiogenesis process. In addition, the flavonoid content in ashitaba leaf nano ethosomal gel has a role in inhibiting the VEGF receptor (VEGFR2) through inhibiting COX-2 activity (Mirossay *et al*., 2017; Hamid *et al*., 2019). This mechanism has been proven in this study, namely the degree of angiogenesis is reduced in the group given 5% ashitaba leaf nano ethosomal gel.

The results of this study are linear with Somwanshi and Hiremath (2018) that the optimal nanoetosomal gel formulation increases collagen synthesis and deposition. The synthesized collagen will be deposited at the wound site and cross-linked to form fibers. Collagen not only provides strength and integrity to the tissue matrix but also plays an important role in homeostasis and epithelialization in subsequent healing phases.

Efficient healing of burn wounds can be linked to adequate collagen production. Considerable collagen synthesis was seen when the dosage of ashitaba leaf nano ethoshomal gel was increased. The findings of this study demonstrate that 5% ashitaba leaf nano ethoshomal gel can promote optimal burn wound healing by increasing collagen synthesis and re-epithelialization through a synergistic method.

## CONCLUSION

In conclusion, burns on rat skin can be effectively treated with transdermal delivery therapy using 5% ashitaba leaf nano ethosomal gel. This method may also be used as a clinical therapy for burn wounds.

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