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Synthesis of Silver Nanoparticles from Lemongrass Leaves Induced Wound Healing by Reduction ROS Fibroblasts

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Abstract—A wound is damaged tissue caused by injury or disease. Meanwhile, fibroblasts are cells found in connective tissues that synthesize, deposit, and remodel the extracellular matrix during wound healing and the imbalance between the ³oxidatives and antioxidants, which contribute to increased reactive oxygen species (ROS). Generally, increased ROS production leads to progressive oxidative damage that slows healing and causes cell death. However, the synthesis of silver nanoparticles with *Cymbopogon citratus* (Lemongrass) extract (NPsCc) decreases ROS production. Therefore, the purpose of this study was to observe the effect of silver nanoparticles extracted from *C. citratus* on ROS fibroblast production. The study was divided into ten groups, namely without treatment, ascorbic acid, hydrogen peroxide, *C. citratus* leaves extract, and five groups of ³silver nanoparticles with *C. citratus* (NPsCc) concentrations of ⁵5 ppm, 20 ppm, 10 ppm, 5 ppm, and 2.5 ppm. Subsequently, the results showed that the 10 ppm NPsCc group decreased ROS fibroblast production compared to the negative control and other NPsCc groups. This was indicated by the percentage of cells with low green fluorescence cell intensity of 48.37%.

Keywords—silver nanoparticles, *C. citratus* leaves extract, reactive oxygen species (ROS), fibroblasts

I. INTRODUCTION

A wound is damaged tissue caused by an injury or disease[1]. In dentistry, oral mucosa wounds are due to brushing mistakes, bites during meals, accidents, or operator errors during dental treatment. However, unhealed wounds produce inflammatory mediators that cause pain, swelling, and infection around the affected site. The pain causes discomfort, difficulty in chewing, and the body experiences the wound healing process. Wound healing is a complex cellular and biochemical process that restores the integrity and function of tissue[2], [3]. It is divided into inflammation, proliferation, and maturation phases[4]. The inflammatory phase aims to remove damaged tissues and prevent infection[5]. Conversely, proliferation is characterized by angiogenesis, a process of forming new blood vessels[3], while the maturation or remodeling phase reshapes the new tissue [6].

¹During the inflammatory phase, neutrophils and macrophages migrate to the injured tissue and produce Reactive Oxygen Species (ROS)[5], [7] in response to bacterial invasion[8], [9]. Excessive ROS levels cause oxidative stress due to an imbalance between prooxidants and endogenous antioxidants in the body[9]. Meanwhile, antioxidants play an essential role in reducing excessive ROS production, decreasing oxidative stress and inflammation during wound healing, while prooxidants do the opposite. Generally, fibroblasts are cells found in connective tissues,

³which synthesize, deposition, and remodeling the extracellular matrix (ECM) during the wound healing process [5]. Also, it plays a role in synthesizing essential substances such as glycosaminoglycans (GAGs), proteoglycans (PGs), glycoprotein multi adhesive, and proteins (collagen, elastin, and reticular) [10]. The disruption of ROS production during healing inhibits fibroblast proliferation and produces an extracellular matrix, which also hinders the process. Therefore, having a natural material, such as silver particles, that reduces the oxidative stress of fibroblasts to improve healing is necessary [11].

Silver particles aid wound healing, including chronic wounds and burns [12]. It has unique properties with efficient reduction, good catalysis, antimicrobial agents, and extensive application. Recent advances in nanotechnology have made strong impacts in many fields, including biomedical. The synthesis of silver nanoparticles is performed by physical, chemical, and biological methods. However, the physical and chemical methods are costly, complex, and environmentally unfriendly because of their toxicity [13]. Subsequently, the results showed that the 10 ppm NPsCc group decreased ROS fibroblast production compared to the negative control and other NPsCc groups. This was indicated by the percentage of cells with low green fluorescence cell intensity of 48.37%. The most widely used natural ingredient for nanoparticle synthesis involves green and fast methods due to their wide distribution, easy and safe handling requirements, and metabolite content necessary for their reduction and stabilization[14]. In addition, the green synthesis of silver nanoparticles promotes wound healing due to its antioxidant activity. This synthesis reduces Ag^+ to Ag^0 and prevents necrosis in wounds by regulating TNF- α expression levels[15].

The synthesis of silver nanoparticles with *C. citratus* decreases toxicity in normal cells and [16]increases its biological effectiveness for antimicrobial[17], anticancer, wound healing, and antioxidant activities [18]. Increased ROS production during wound healing can be suppressed with silver nanoparticles from *C. citratus* leaf extract, which produces a less reactive free radical. Therefore, this ³search was performed to determine the in vitro influence of silver nanoparticles from ethanol extracts of *C. citratus* leaves on the production of ROS fibroblasts associated with wound healing. Subsequently, these components incorporated the applicable criteria.

II. MATERIAL AND METHODS

A. Study design

This research was an in vitro experiment conducted in the Faculty of Dentistry, Trisakti University, Jakarta, Indonesia.

B. Preparation of *Cymbopogon citratus* Leaves Extract

C. citratus leaves were obtained from Bogor, West Java, Indonesia. The leaves were extracted using 70% ethanol in a ratio of 1:1 at room temperature, then shuffled manually for three days. Then, the extract was filtered using Whatman filter paper and rotated using an evaporator at 50-60°C. Finally, phytochemical tests were conducted on the extract using 18-16-2/MU/SMM-SIG (LCMS/MS) QTOF [19].

C. Preparation of Silver Nanoparticles of *Cymbopogon citratus* Extract

About 500 mg of extracted *C. citratus* leaves were dissolved in 100 mL of water, then mixed with 90 ml of 0.1 M AgNO₃ (Merck). The solution mixture using a magnetic stirrer at 2500 rpm and heated up to 40°C for 2 hours. Afterward, the solution was incubated for 24 hours in dark conditions (covered with aluminum foil) until the color changed to dark brown, indicating the formation of silver nanoparticles with *C. citratus* (NPsCc). Then, 10 mL of the NPsCc was tested for particle size using a VASCO-2 particle size analyzer (Cordouan Technology, France). The residual solution was transferred into a centrifuge flask and centrifuged at room temperature of 9000 rpm. Subsequently, the black deposits obtained were washed twice with distilled water, then five concentrations of 40 ppm, 20 ppm, 10 ppm, 5 ppm, and 2.5 ppm NPsCc were prepared [20].

D. ROS Fibroblast Test

The fibroblasts were grown in the DMEM cell culture at 37°C and 5% CO₂. Ten groups were used, namely fibroblasts without treatment with silver nanoparticles from *C. citratus* (NPsCc) (K0), positive control as ascorbic acid (K1), and *C. citratus* leaves extract (K2). The others were silver nanoparticles (K3), hydrogen peroxide (K4), and 5 NPsCc groups with concentrations of 40 ppm, 20 ppm, 10 ppm, 5 ppm, and 2.5 ppm incubated for 3 hours before washing the cells with 1X PBS. Hydrogen peroxide and 1 mL of probe 2',7'-dichlorodihydrofluorescein diacetate (H2DCFDA) were added and incubated for 30 minutes. Then, 1 mL of 3 µM 4',6-diamidino-2-phenylindole (DAPI) was added. Green fluorescent cells were observed using Zeiss Z1 inverted Fluorescence (Germany) and calculated with the Image J software. The result of the percentage ROS production was the number of cells with the green fluorescence intensity divided by the total number of cells and multiplied by 100 [21].

III. RESULTS AND DISCUSSION

This study determined the effect of silver nanoparticles synthesized from *C. citratus* leaves extract to improve the stability, solubility, and effectiveness. As shown in Table I, the phytochemical test results indicated the presence of a secondary metabolite acting as an antioxidant.

The synthetic characteristics of the NPsCc leaves extract with a polydispersity index (PDI) of 0.027 showed homogeneous or monodispersed samples [22]. Generally, particle size distribution and poly density index (PDI) play a role in determining physical characteristics during pharmaceutical product development, stability, efficiency, drug release, mucoadhesion, and absorption [23]. Meanwhile, the average NPsCc was 1044 nm, and the NPsCc particle size distribution measurement showed a dv10 value of 851.36 nm, indicating that 10% of the total particles are smaller than 851.36 nm. The dv50 value was 1071.89 nm, meaning 50% had smaller particle size distributions than 1071.89 nm. The

dv90 distribution of NPsCc showed that 90% had sizes below 1288.59 nm. Table II shows the polydispersity index values and particle size distribution of NPsCc.

TABLE I. PHYTOCHEMICAL RESULTS OF *C. CITRATUS*

Number	Active Compounds	Results
1	Alkaloids	Positive
2	Flavonoids	Positive
3	Tannins	Positive
4	Steroids	Positive
5	Triterpenoids	Positive
6	Saponins	Positive

TABLE II. POLYDENSITY INDEX VALUE AND NPsCc PARTICLE SIZE DISTRIBUTION

Num	dv10 (nm)	dv50 (nm)	dv90 (nm)	Z-avg (nm)	PDI
NpsCc	851.36	1071.89	1288.59	1044	0.027

These results of the physical characteristics of NPsCc showed that nanoparticles were obtained, and this follows other studies that group particles in nano-forms have a size of 10-1000 nm. In addition, the NPsCc polydispersity index (PDI) showed that particles were monodispersed and ranged between 0.035-0.05, indicating homogeneous particles. PDI represents the size population distribution in a given sample, with a value of <0.2 acceptable as nanoparticles. Therefore, these particle sizes are suitable for drug production in the pharmaceutical field.

The production of ROS fibroblasts was proven by a 2',7'-dichlorofluorescein diacetate (H2DCFDA) probe, which can penetrate the cell membrane, and is used to detect free radicals that cause oxidative stress. The presence of esterase enzymes results in a biochemical reaction that converts H2DCFDA into a non-fluorescent component of H2DCF. Subsequently, high free radicals (ROS) convert H2DCF into fluorescent 2',7'-dichlorofluorescein (DCF), with a green color. The fluorescence intensity of the H2DCFDA probe provided quantitative information on ROS levels in cells.

ROS is a free radical that mediates intracellular damage to lipids, proteins, carbohydrates, and nucleic acids. ROS is highly reactive because of unpaired electrons, which cause unstable conditions, resulting in oxidative stress due to an imbalance between oxidant molecules and antioxidants. Therefore, it can destroy the internal redox balance, causing tissue damage. ROS is created as a by-product during oxidative metabolism, i.e., reactive species, such as superoxide anions (O₂⁻), hydrogen peroxide (H₂O₂), and hydroxyl radicals (OH), which are radical and nonradical oxygen derivatives, produced by the partial reduction of oxygen [24], [25].

The calculation results using Shapiro-Wilk showed the data had a value of p>0.05. Testing continued with the one-way ANOVA parametric test, where the groups showed a significant difference at p<0.05. The Duncan test result revealed that the group without NPsCc treatment and stressor (K0) produced a green fluorescence cell intensity of 54.76% and showed a significant difference (p<0.05) with groups K1, K4, P2, and P3. Subsequently, the fluorescence intensity in groups K1, P2, and P3 were higher than in group K0, while K4 was higher. The group given ascorbic acid as a positive control (K1) had a green fluorescence cell intensity of 46.13% and showed a significant difference (p<0.05) with groups K0, K3, K4, P5, and P1. However, their intensity were higher than in

the K1 group. Groups K2, P4, P3, and P2 showed no significant difference ($p>0.05$) with group K1. The fluorescence intensity of these groups were slightly higher than K1 but lower than K0, K3, K4, P5, and P1.

Furthermore, the comparison group given *C. citratus* leaves extract (K2) had a green fluorescence cell intensity of 52.04% and showed a significant difference ($p<0.05$) with groups K4 and P1. However, the intensity in group P4 and P1 were higher compared to group K2. Although K2 showed no significant difference ($p>0.05$) with K1, P2, P3, and P4 groups, its fluorescence intensity was slightly higher than the groups K3 and P5 were not significantly different, but their intensity were higher than group K2.

In the other comparison group, the silver nanoparticles (K3) had an intensity of 52.22%, showed a significant difference with groups K1, K4, and P1. The intensity of green fluorescent cells in groups K4 and P1 was higher, while group K1 was lower than groups K4. In addition, the comparison group given hydrogen peroxide stressor (K4) had a green fluorescence cell intensity of 61.72%, which showed a significant difference ($p<0.05$) with other groups. Meanwhile, the intensity of the green fluorescence cells in group K3 was higher compared to other groups. Table III shows the average intensity of green fluorescence cells in all groups based on the one-way ANOVA and Duncan tests.

The NPsCc treatment in groups P1, P2, P3, P4, and P5 showed that P3 had the lowest green fluorescence cell intensity at 48.37%, followed by groups P2, P4, P5, and P1 at 48.54%, 49.39%, 55.18%, and 59.19%. P3 group showed a significant difference ($p<0.05$) with groups P1 and P5, whose intensity were higher. However, P2 and P4 groups showed no significant difference ($p>0.05$) with P3 groups, and their intensity were also slightly higher.

Generally, ROS plays several roles during the wound healing process. First, it regulates the narrowing of blood vessels (vasoconstriction) and induces thrombus formation by platelets. Second, ROS signaling causes rapid migration of neutrophils and macrophages from the local blood vessels to the injury site during lymphocyte recruitment. Third, in the defense against pathogenic microorganisms, ROS kill bacteria and fungi through phagocytosis by the release of bacteriostatic H_2O_2 from platelets and neutrophils. Fourth, ROS mediates cell division and migration of keratinocytes, endothelial (angiogenesis), and fibroblasts in collagen formation during tissue repair. The commonly produced free ROS radical during wound healing is H_2O_2 . It is a reactive molecule synthesized by various cells that influences potential membrane changes, the formation of new molecules, and changes in intracellular redox balance. These actions result in the activation or inactivation of different signal transduction pathways[26], [27].

According to the microscopic observations, the fibroblast of group K0 naturally produced ROS, while hydrogen peroxide stressors showed an increase in fibroblast production in all groups. Meanwhile, the antioxidant administration using ascorbic acid showed a significant decrease compared to other groups. In contrast, the groups with *C. citratus* leaves extract and silver nanoparticles demonstrated a reduction in the intensity of green fluorescence cells. Also, the synthesis of silver nanoparticles from *C. citratus* leaves extract decreased ROS production in some treatment groups. The results of NPsCc treatment at varying concentrations showed that the 10

ppm group caused greater ROS reduction compared to other NPsCc groups, indicated by a low fluorescence cell intensity. Fig. 1 shows an overview of the green and blue fluorescence cell intensity in the groups.

Furthermore, inducing H_2O_2 in the groups increased ROS production in the comparison group given this treatment, causing cellular damage. The Fenton reaction between H_2O_2 and Fe^{2+} ions acts as an oxidative stressor trigger [28] and generates a highly reactive hydroxyl radical (OH) as the primary mechanism of oxidative damage [29]. However, non-enzymatic antioxidants such as ascorbic acid decrease ROS production, resulting in low-intensity fluorescence cells. Ascorbic acid or vitamin C is a popular antioxidant supplement, which neutralizes oxidative stress through the donation or transfer of electrons. It also scavenges superoxide (O_2^-), hydroxyl (OH), and hydrogen peroxide (H_2O_2) radicals. In addition, exogenous ascorbic acid reduces free radicals to inhibit lipid peroxidation and prevent cell damage [30].

In addition, the comparison group given silver particles or *C. citratus* leaves extract after the induction of H_2O_2 showed a decrease in ROS production. Silver particles regenerate reactive oxygen species (ROS)[11] which trigger an antioxidant system that lowers ROS levels. The group with *C. citratus* leaves extract exhibited a decrease in the intensity of green fluorescence cells. This proved that the antioxidant contents, such as alkaloids, flavonoids, tannins, steroids, saponins and triterpenoids[31], act as non-enzymatic antioxidants and are able at lowering ROS production. Antioxidants apprehend and neutralize free radicals and prevent damage caused to normal cells, proteins, and fats. Antioxidants have a molecular structure that transfers electrons to free radical molecules without impaired function, thereby breaking its chain reaction [32].

The groups given NPsCc treatment showed decreased ROS production, with low intensity of green fluorescence cells after induced stressors. Generally, the biological synthesis of silver particles with *C. citratus* leaves reduces agent and capping, as well as improves stability and non-toxicity [33]. The synthesis of silver nanoparticles with natural ingredients, such as *C. citratus* leaves extract, acts as antioxidants to reduce oxidative stress and trigger protective mechanisms for tissue integrity and structure improvement. Therefore, it plays a role in the wound healing process by decreasing ROS production[34].

According to Keerthiga et al.[35], silver nanoparticles synthesized with *C. cyminum* oil extract act as antioxidants and have good wound healing effects. The free radical exterminator accelerates the inflammatory stage, forms blood vessels and collagen matrix, alters membrane potential, and inhibits ATP enzyme synthesis resulting in faster skin regeneration and wound healing. Layeghi-Ghalehsoukhteh et al.[36], stated that gold nanoparticles of *Tragopogon dubius* leaves extract have potential in wound healing due to their antioxidant properties. Meanwhile, the best NPsCc concentration that decreased ROS production was 10 ppm. Therefore, silver nanoparticles should not be more than 40 ppm in normal cells as they result in toxicity and cause apoptosis. The synthesis of silver particles from *C. citratus* leaf extract reduces oxidative stress, enabling fibroblasts to proliferate and synthesize extra cell matrices such as collagen and basic substances help accelerate wound healing [12].

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TABLE III. AVERAGE ROS PRODUCTION BASED ON THE PERCENTAGE OF GREEN FLUORESCENT CELLS

Groups	Number of Samples (n)	Green Fluorescent Intensity (%)	p-value
Fibroblasts	3	54.76 ±1.54 ^{bc}	0.000
Ascorbic Acid	3	46.13 ±2.56 ^c	
<i>C. citratus</i> Leaves	3	52.04 ±4.12 ^{cde}	
Silver Nanoparticles	3	52.22 ±3.89 ^{cd}	
Hydrogen Peroxide	3	61.72 ±0.60 ^a	
NPsCc 2.5 ppm	3	55.18 ±2.35 ^{bc}	
NPsCc 5 ppm	3	49.39 ±3.10 ^{cde}	
NPsCc 10 ppm	3	48.37 ±4.03 ^{de}	
NPsCc 20 ppm	3	48.54 ±2.68 ^{de}	
NPsCc 40 ppm	3	59.19±4.68 ^{ab}	

^{a-c} In different columns showed significant differences ($p < 0.05$)

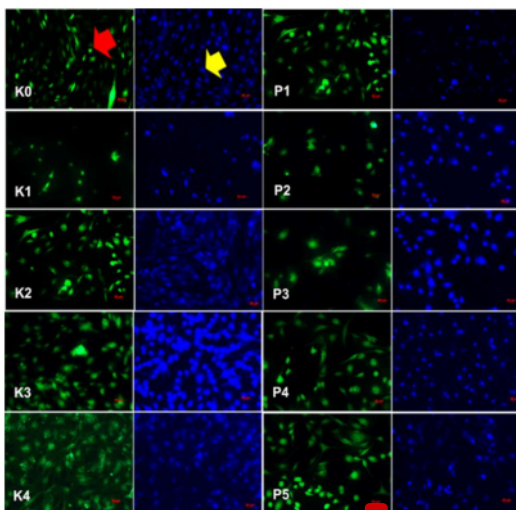


Fig. 1. The effect of NPsCc administration on the production of ROS fibroblasts, measured using the H2DCFDA probe (green) and DAPI probe (blue) to calculate the number of cells. The red arrow shows ROS fibroblast with green fluorescence intensity, while the yellow arrow indicates the fibroblast cores colored blue. The figure shows the green fluorescence intensity in the untreated (K0), ascorbic acid (K1), *C. citratus* leaves extract (K2), nanoparticles silver (K3), and hydrogen peroxide (K4) groups. The groups given NPsCc at concentrations of 40 (P1), 20 (P2), 10 (P3), 5 (P4), and 2.5 ppm (P5) are also exhibited. The observation was at 100X magnification.

IV. CONCLUSION

The study results showed that the synthesis of silver nanoparticles from ethanol extract of *C. citratus* leaves effectively reduces the production of Reactive Oxygen Species by fibroblasts, causing a low green fluorescence intensity. Meanwhile, the fibroblast cell intensity was 48.37%, visible at a concentration of 10 ppm. Therefore, antioxidant production helps wound healing by preventing the oxidation process caused by free radicals.

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