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The antioxidant activity of lemongrass leaves extract against fibroblasts oxidative stress

Atividade antioxidante do extrato das folhas de capim-limão contra o estresse oxidativo em fibroblastos

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ABSTRACT

Objective: The purpose of this research is to assess the antioxidant activity of lemongrass leaves extract in terms of lowering ROS generation and its effect on the viability and proliferation of fibroblasts under oxidative stress. **Material and Methods**: The antioxidant activity was measured using the DPPH method and the ROS assay was carried out by fluorescent H2DCFDA staining. Viability and proliferation assays were performed using the Cell Counting Kit-8 (CCK-8) and was read at 450 nm using microplate reader. The groups were divided into 8, namely fibroblasts without treatment (comparison group), fibroblast induced by H_2O_2 (negative control), fibroblast with H_2O_2 then treated with ascorbic acid (positive control), and fibroblast with H_2O_2 then treated with lemongrass leaves extract at various concentrations (10, 20, 30, 40, and 50 ppm). **Results**: The results showed that the antioxidant activity of lemongrass leaves extract had an IC_{50} value of 64.17 ppm. ROS production were reduced by the LgLE of all concentrations if compared with negative control ($p=0.819$). LgLE can maintained the fibroblast viability with 10 ppm of LgLE was the most optimum concentration ($p<0.05$). LgLE can induced the proliferation of fibroblast, with the most effective was at 24 h of observation ($p<0.05$). **Conclusion**: Lemongrass leaves extract has a strong antioxidant activity that can reduce oxidative stress and increase the viability and proliferation of fibroblasts with the optimum concentration is at 10 ppm.

KEYWORDS

Antioxidant activity; Fibroblast; Lemongrass; Oxidative stress; Reactive oxygen species.

RESUMO

Objetivo: O intuito deste estudo foi determinar a ação antioxidante do extrato das folhas de capim-limão no que se refere a diminuição da produção de espécies reativas do oxigênio (EROS) e o seu efeito na viabilidade e proliferação de fibroblastos submetidos à estresse oxidativo. **Material e Métodos**: A atividade antioxidante foi medida utilizando o método de DPPH e o ensaio de EROS foi realizado pela coloração fluorescente de H2DCFDA. Os ensaios de proliferação e viabilidade foram realizados utilizando-se o kit de contagem de células CCK-8 em microplacas de leitura à 450nm. Os grupos foram divididos em 8: Fibroblastos sem tratamento (grupo controle), Fibroblastos tratados com H2O2 (controle negativo), Fibroblastos tratados com H2O2 e extrato da folha de capim-limão em concentrações variadas (10, 20, 30, 40 e 50 ppm). **Resultados**: Os resultados mostraram que a atividade antioxidante do extrato de capim-limão teve uma IC50 (com o numeroal subscrito) com valor de 64.17ppm. A produção de ROS foi reduzida pelo tratamento com o extrato em todas as concentrações testadas quando comparado ao grupo controle negativo ($p=0.819$). O extrato manteve a viabilidade dos fibroblastos, sendo 10ppm a concentração menos tóxica ($p<0.05$). LgLE pôde induzir a proliferação de fibroblastos, sendo que a melhor eficiencia foi após 24h de observação ($p<0.05$). **Conclusão**: O extrato das folhas de capim-limão apresentam forte atividade antioxidante reduzindo o estresse oxidativo e aumentando a viabilidade e proliferação de fibroblastos, sendo a concentração ótima de 10ppm.

PALAVRAS-CHAVE

Atividade antioxidante; Fibroblastos; Capim-limão; Estresse oxidativo; Espécie reativa de oxigênio.

INTRODUCTION

Wounds or injuries in the oral soft tissues are very common and often encountered in daily life that can be caused by physical, chemical, or thermal trauma [1]. The wound healing process consists of 4 interconnected phases namely hemostasis, inflammation, proliferation, and remodelling phase. During the inflammatory phase, phagocytic cells such as neutrophils and macrophages will invade the injured area with the aim of killing microorganisms and decontaminating the wound area [2]. Neutrophils and macrophages will also experience a *respiratory burst,* which is an increase in oxygen use and will produce an excessive amounts of Reactive Oxygen Species (ROS), like superoxide and hydrogen peroxide. These species have a beneficial role in microbial killing and also intracellular signalling such as inducing vascular endothelial growth factor (VEGF) in order to enhance the angiogenesis process [3]. At a balanced amounts, ROS has beneficial effects on several physiological processes of the body including wound healing, regulating oxidation-reduction reactions, and invading the pathogens [4]. But if ROS exceeded that which the body can handle, it can lead to a shifted homeostasis, and causing cells to experience an oxidative stress.

Oxidative stress occured when there is an elevated production of ROS and at the same time the antioxidant response is slightly reduced because of the excess oxidants, causing an imbalance amounts between the number of prooxidants and antioxidants in cells [5]. Furthermore, oxidative stress interferes with the communication function between cells and causes tissue damage, thereby disrupting the wound healing process, such as prolonging the inflammatory phase and inhibiting the proliferation process and collagen formation by fibroblasts [6]. Our body has a defense mechanism against oxidative stress caused by the increase production of ROS, namely with endogenous enzymatic antioxidants, such as glutathione peroxidase, superoxide dismutase (SOD), glutathione, catalase (CAT), uric acid, lymphoid acid, and coenzymes, and non enzymatic antioxidants such as vitamins C and E, carotenoids, and flavonoids, which are all can be obtained from diet like fruits, nuts, and vegetables [7]. Antioxidants mechanism of action in fighting free radicals is by breaking the reaction chain, chelating metals that act as a pro-oxidants, as well as capturing aggressive ROS such as superoxide and H_2O_2 [8]. However, in a wound healing state, the production rate of antioxidants is often not proportional to ROS production [9,10]. Therefore, natural exogenous antioxidants with good biocompatibility, biodegradability, and physicochemical bonds are required to help balancing ROS production, such as lemongrass *(Cymbopogon citratus)*.

Cymbopogon citratus or commonly called as lemongrass, is a tropical perrenial plant that belong to family Poaceae. Lemongrass is popular as a cooking spice especially in Indonesia, mostly the stem part, hence, the leaves often become a waste [11]. Surprisingly, lemongrass leaves have essential oils that contain citral, aldehyde, geranial, and neral, which are often processed into aromatherapy [12]. in addition to essential oils, lemongrass leaves also included active components such as flavonoids, saponins, tannins, phenols, phenolic acids, and terpenoids [13]. which the antioxidant contents are higher than the stems [14]. Furthermore, the leaves have various pharmacological activities such as antibacterial, antifungal, and antidiarrheal [12], as well as act as an anti-inflammatory and antioxidant that can enhancing the wound healing process, but has not been studied thoroughly. Thus, the purpose of this study is to investigate the antioxidant activity of lemongrass leaves extract (LgLE) in order to decrease the ROS production and increase the viability and proliferation rate of fibroblasts under oxidative stress, which conditioned during the wound healing process.

MATERIAL AND METHODS

Preparation of lemongrass leaves ethanol extract

Fresh lemongrass leaves that were dried naturally for 2 weeks were collected from Parung area, Bogor, West Java. The sample was extracted for 24 h at room temperature using a 70% ethanol as a solvent (Merck) at a ratio of 1:10 (w/v). The macerate was then filtered through filter paper and evaporated for 2 h at 40°C in a rotary vacuum evaporator (Buchi R-215, Germany) until a pure and thick LgLE was produced [11,15].

Antioxidant assay

The antioxidant activity was measured by 2,2-diphenyl-1-picrylhydrazil (DPPH) radical scavenging activity (Sigma Aldrich, Missouri,

USA). In a volumetric flask, 4 mg of DPPH was dissolved in 25 mL of methanol (Merck, Darmstadt, Germany) to provide a solution with a concentration of 160 ppm. The solution was then stored in a dark room and covered with aluminum foil. The extract samples, methanol, and DPPH solution were then put in a test tube and kept at room temperature for 30 min. The absorbances were measured using a UV-Vis spectrophotometer at 517 nm (UV-1800 Shimadzu, Japan).

Fibroblast culture

In the present study, Human Dermal Fibroblasts were tested and obtained from Biorepository of Stem Cell Research Center, Yarsi University, Indonesia. For 24 h, fibroblasts were cultivated and cultured at 37°C in a 5% CO_2 environment. Following that, the fibroblast growth medium was changed with Dulbecco's modified Eagle's medium (DMEM) enriched with 10% Fetal bovine serum (FBS) (Gibco) and antimycotic-antibiotics drugs such as penicillin, streptomycin, and amphotericin B, then the cells were divided into eight groups. Seven of the eight groups were then subjected to oxidative stress by adding 100 μM of H_2O_2 as stressor and then incubated for 60 min. for more specific, the first group is fibroblasts without any treatment as a comparison group; the 2nd group was only treated with H_2O_2 100 μM as negative control; the 3rd group was treated with H_2O_2 100 μM and ascorbic acid as positive control, and the remaining groups were treated with H_2O_2 100 μM and various concentrations of LgLE (10;20;30;40;50 ppm).

ROS assay

Fibroblasts were incubated for 24 h after being plated in a triplicate cell culture dishes at a density of 10 x 10^3 cells per dish. The medium was then replaced with a solution of LgLE (10, 20, 30, 40, and 50 ppm), ascorbic acid as positive control, and DMEM for the comparison group and negative control group, then incubated for another 3 h. The dishes were then rinsed with PBS 1X before loading 10 μM of H2DCF-DA reagent (Santa Cruz, USA) into each dish and incubating them at 37°C in dark for 30 min. The groups were rinsed twice using PBS 1X, followed by nuclei staining using Hoechst 33342 (Invitrogen, Thermo Fisher Scientific, USA) at a concentration of 5 ppm for 30 min at 37°C in dark. After staining, all groups were thrice washed and then observed using EVOS Fluorescent Microscope (Invitrogen, Thermo Fisher Scientific, USA). FIJI ImageJ Software was used to count the cells. The fraction of cells can be counted by dividing the quantity of cells fluoresced green by the total of cells (blue fluoresence) and then multiplied by 100.

Cell viability and proliferation assay

The cell viability and proliferation assay were carried out as described previously by Che Zain et al. [16], with a minor modification. Fibroblasts were seeded on a 96-well plate at a density of 10 x 10^3 per well and incubated at 37°C for 24 h. After adhered to the surface of the wells, the medium was replaced with 200 μL of varied concentrations of sample solutions and ascorbic acid for the control. At the indicated times, the treatments were discarded, and the wells were washed with PBS 1X. Following that, 100 μL of CCK-8 solution (Dojindo Lab, Kumamoto, Japan) was injected into each well and incubated for another 90 min. A Microplate reader was used to measure absorbance at 450 nm (Tecan Group Ltd., Mannedorf, Switzerland). Experiments were carried out in triplicate. The percentage of living cells were calculated using the following equation:

$$\%\text{cells' viability} = \frac{\text{absorbance of treated group}}{\text{absorbance of control}} \times 100 \quad (1)$$

The percentage of proliferation can be calculated by the formula:

$$\%\text{proliferation rate} = \frac{\text{absorbance of sample}}{\text{absorbance of negative control}} \times 100 \quad (2)$$

Data analysis

Statistical quantification was performed using SPSS with version 2.3. The data is presented as mean ± standard deviation (SD). To verify normalcy, the Shapiro-Wilk test was performed. If the findings indicated a normal data ($p > 0.05$), one-way ANOVA was used for the ROS and viability assays, while MANOVA was used for the proliferation assay, if a significant difference ($p < 0.05$) was found using ANOVA or MANOVA, the Post Hoc's Tukey test was performed.

RESULT

This study was done to determine the effect of LgLE, which was macerated using 70% ethanol as a solvent. The described solvent was used because of its polar properties that can

attracted more bioactive components contained in lemongrass leaves compared to the semipolar and non-polar. Thus, it is expected to have higher antioxidant activity [17].

Fibroblast oxidative stress was simulated by exposing fibroblasts with H_2O_2 solution for 1 hour [18]. The generation of ROS in fibroblasts was proven using the H2DCFDA probe, which is an instrument used to detect free radicals, such as H_2O_2, O_2, OH, and NO [19]. The H2DCFDA probe may easily pass through the cellular membrane and react to esterase enzymes, resulting in biochemical process that transforms H2DCFDA to a non-fluorescent molecule H2DCF. In the presence of large quantities of ROS inside the cell membrane, H2DCF becomes oxidized and converted to 2′,7′-dichlorofluorescein (DCF), resulting in green fluorescences, and their intensity were calculated quantitatively to determine the presence of ROS and their level inside cells [20,21].

The effect of LgLE on reducing fibroblast oxidative stress is determined by examining the level of ROS formation and also the ability of fibroblasts to survive and multiply during the wound healing process [22]. The study was divided into eight groups: fibroblasts without treatment (comparison), fibroblasts generated by hydrogen peroxide (H_2O_2) as a negative control, fibroblasts administered ascorbic acid as a positive control, and the groups that were treated with LgLE at a concentration of 10, 20, 30, 40, and 50 ppm. Cells viability and proliferation were evaluated using CCK-8/WST-8 assay, which showed the survival ability of fibroblasts, with the reduction of formazan dye by dehydrogenase activites in cells to give a formation of yellow-colored dye.

Antioxidant activity of lemongrass leaves extract

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) method was used for antioxidant activity. This method measured the absorbances of antioxidant activity quantitatively using UV-Vis spectrophotometer and the value of free radical scavenging activity was determined and expressed with the IC_{50} (Inhibitory Concentration) value, that is a concentration of the tested compound that was needed to reduce free radical DPPH by 50% [23]. The antioxidant data results of LgLE with concentrations of 10, 20, 30, 40, and 50 ppm showed that the average percentage of extract inhibition was 7.08%, 14.01%, 25.19%, 31.64%, and 37.68%, respectively. The percentage of inhibition is shown in Table I. The result shown in Table I is used to determine the IC_{50} value of LgLE, which was 64.17 ppm. The IC_{50} value was then calculated from the average results of the linear regression equation from LgLe in 3 repeated experiments. These results are shown in Figure 1.

Table I - the percentage of inhibition and IC_{50} value of LgLE

Lemongrass Leaves extract (ppm)	inhibition (%)			Mean SD	IC_{50} (ppm)
	1	2	3		
10	7.08	3.37	10.78	7.08 ± 3.70	
20	14.68	12.32	15.03	14.01 ± 1.48	
30	27.10	24.00	24.46	25.19 ± 1.68	64.17 ± 2.94
40	31.21	29.16	32.64	31.64 ± 1.75	
50	39.53	35.26	38.24	37.68 ± 2.19	

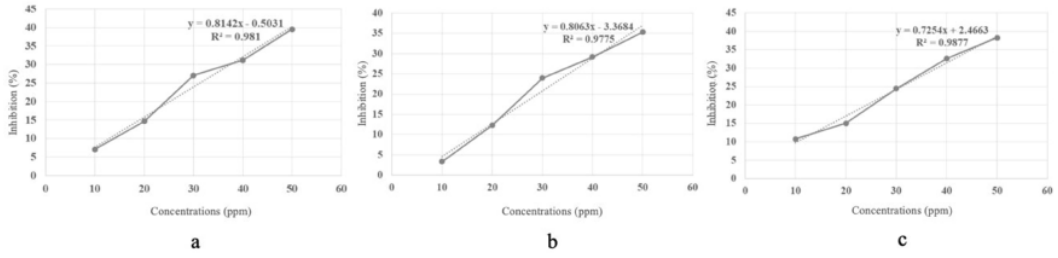

Figure 1 - The relationship curve of the LgLE concentrations to the percentage of inhibition on a. 1st repetition; b. 2nd repetition; c. 3rd repetition.

Production of ROS

Under a fluorescent microscope, the formation of ROS was identified by recognizing the green fluorescence of fibroblasts. Statistical analysis revealed no significant difference ($p = 0.819$) between the study groups (Table II). However, in each group the intensity of green fluorescent cells was different. The group exposed with H_2O_2 (negative control) showed a higher number of green fluorescent cells compared to others, meaning that the ROS production was remarkably elevated. The groups that were treated with LgLE of 50, 40, and 30 ppm had a lower intensity of green fluorescent cells compared to negative control. Meanwhile the 20 ppm and 10 ppm of LgLE had a much lower intensity of green fluorescent cells and could bring it down almost nearly to the comparison group and positive control group.

Moreover, group treated with a 10 ppm concentration of LgLE showed a better ability to reduce fibroblast oxidative stress compared to the comparison groups. However, the intensity of green fluorescent cells in the group treated with 10 ppm concentration had a slightly higher intensity than the ascorbic acid group. Meanwhile, the intensity of green fluorescent cells for the positive control group was lower than all the other groups. The intensity of green fluorescent cells in each group is shown in Figure 2.

Viability of fibroblasts

The test results showed that the 10 ppm of LgLE group had a viability value 3.17% higher compared to comparison group, and 0,77% lower than positive control group although not significantly different ($p>0.05$). The group that was exposed with H_2O_2 only (negative control) with a viability value of 68.33% showed a significant difference ($p<0.05$) with the comparison group and positive control group, as well with the 10 ppm of LgLE. The result of the viability assay is shown in Table III.

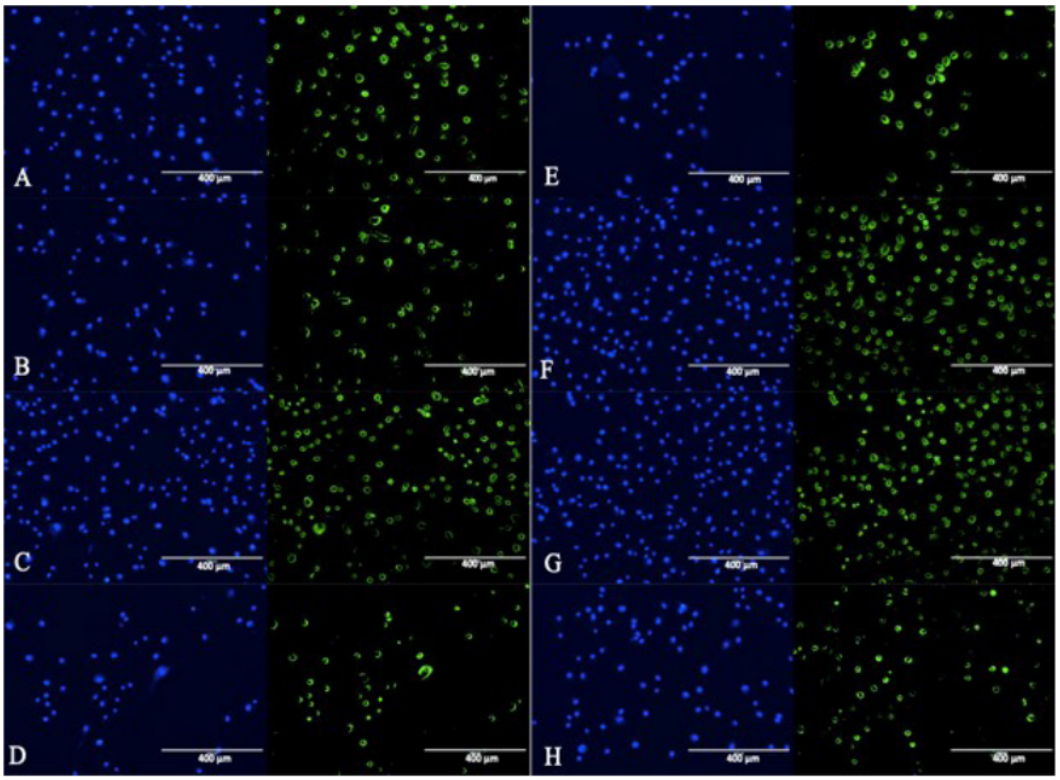

Figure 2 - The impact of LgLE on ROS generation in fibroblasts as evaluated by the H2DCF-DA probe (green) and total cell count as determined by the Hoechst probe (blue); A. Green fluorescence cells in the comparison group; B. Negative control group (H_2O_2 induced only); C. Positive control group (ascorbic acid); D. Group with 10 ppm of LgLE; E. 20 ppm; F. 30 ppm; G. 40 ppm; H. 50 ppm. Observations at 100X magnification.

Table II - ROS production as a fraction of the intensity of green fluorescent cells

groups	Number of samples (N)	Green fluorescent intensity (%)	*p value*
Fibroblast (comparison)	3	44.57 ± 4.18	0.819
Negative control	3	55.72 ± 6.31	
Positive control	3	38.19 ± 21.06	
LgLE 10 ppm	3	41.69 ± 9.36	
LgLE 20 ppm	3	44.45 ± 17.01	
LgLE 30 ppm	3	45.50 ± 9.10	
LgLE 40 ppm	3	47.80 ± 11.12	
LgLE 50 ppm	3	48.58 ± 14.19	

Table III - Viability of Fibroblasts

groups	Number of samples (N)	Mean ± SD (%)	*p Value*
Fibroblast (comparison)	3	84.33 ± 3.51^{ab}	p<0.05
Negative control	3	68.33 ± 7.64^{c}	
Positive control	3	87.67 ± 1.53^{a}	
LgLE 10 ppm	3	87.00 ± 4.36^{a}	
LgLE 20 ppm	3	75.00 ± 2.00^{bc}	
LgLE 30 ppm	3	72.33 ± 0.58^{c}	
LgLE 40 ppm	3	70.00 ± 2.64^{c}	
LgLE 50 ppm	3	69.33 ± 1.53^{c}	

$^{a-c}$ in various columns indicate significant differences (p<0,05)

There was no significant difference (p>0.05) between the LgLE with concentrations of 20, 30, 40, and 50 ppm groups and negative control group, but the LgLE groups showed a higher viability value by about 9.76%, 5.86%, 2.44%, and 1.46%, respectively, compared with negative control. The microscopic observation on the viability of fibroblasts in all study groups showed a good cell condition that could still survived and also that undergone an apoptosis. These results with 100x magnification are shown in Figure 3.

Proliferation of fibroblasts

The fibroblasts proliferation rate were observed three times at 24 h, 48 h, and 72 h. The 24 h observation showed that the proliferation of comparison group was significantly (p<0.05) higher compared with negative control and LgLE groups of 40 ppm and 50 ppm. The group given LgLE of 10 ppm showed a significant difference (p<0.05) between the negative control, as well as with 30, 40, and 50 ppm of LgLE groups. Meanwhile, for the comparison group and positive control group, the 10 ppm of LgLE didnt show a significant difference (p>0.05) but had a higher proliferation rate around 3,53% than comparison group, and slightly lower proliferation rate by about 0,86% compared with positive control, meaning that the proliferation rate of 10 ppm of LgLE was almost the same with the ascorbic acid. The observation results at 24 h showed the proliferation of fibroblasts from the highest to lowest was the positive control, 10 ppm of LgLE, comparison group, LgLE groups with 20 ppm, 30 ppm, 40 ppm, and 50 ppm, and negative control group.

The 48 h observation showed the comparison group is significantly different (p<0.05) with negative control group and groups that were given the LgLE at 20, 30, 40, and 50 ppm. The negative control group differed significantly (p<0.05) from the comparison group positive control, as well with LgLE at 10 ppm. There was also a significant difference (p<0.05) between the group given LgLE with a concentration of 10 ppm and negative control and LgLE with concentrations of 20, 30, 40, and 50 ppm. The 10 ppm of LgLE at 48 h showed a fairly high

proliferation compared to other groups, and also was higher by 2.1% than the positive control group. At 48 h of observation, the highest to lowest proliferation rate was the 10 ppm of LgLE group, positive control group, comparison group, group that were given LgLE at a concentration of 20 ppm, 40 ppm, and 30 ppm respectively, negative control group and lastly the 50 ppm of LgLE group (Table IV).

At 72 h of observation, there was a significant difference ($p<0.05$) between the negative control group and 10 ppm of LgLE group. The 10 ppm of LgLE group also showed a significant difference ($p<0.05$) with the LgLE group of 20, 30, 40, and 50 ppm. The 10 ppm of LgLE did not show a significant difference ($p>0.05$) with the comparison group and positive control group, but the proliferation rate was 23.8% and 48.5% higher, respectively. The comparison group was also had a slightly higher proliferation rate compared with positive control group. At 72 h of observation, the highest to the lowest proliferation rate was the 10 ppm of LgLE, comparison group, positive control group, 20 ppm, 30 ppm, and 40 ppm of LgLE groups, negative control group, and 50 ppm of LgLE group.

The proliferation rate of fibroblasts group observed between 24, 48 and 72 h were significantly different ($p<0.05$). In negative control group, the 24 h observation was significantly different from 48 h and 72 h, where the proliferation rate at 48 h is higher than the 72 h. The positive control group and the 10, 20, and 40 ppm of LgLE groups showed a significant difference ($p<0.05$) between the 24 h, 48 h, and 72 h of observation, where it implies that the longer observation time resulting in a lower proliferation rate. However, 10 ppm of LgLE at 72 h observation was able

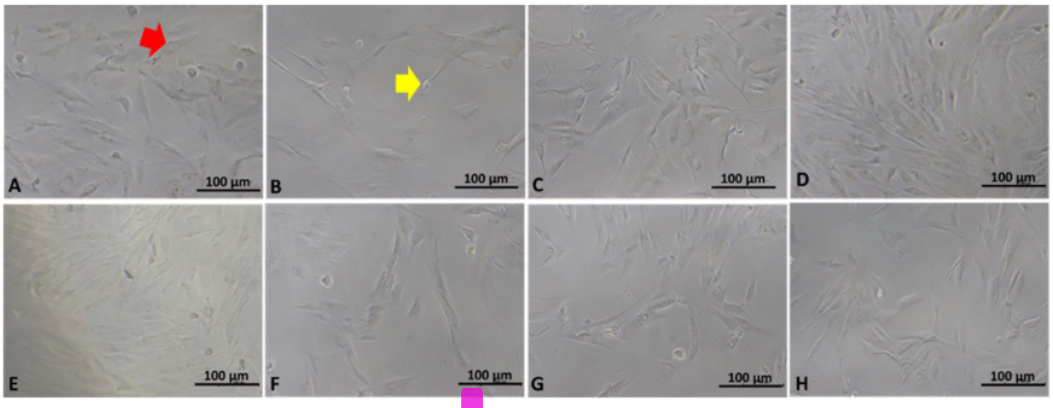

Figure 3 - Effect of LgLE on the viability of fibroblasts. Red arrows show live fibroblasts with clearly visible cell nuclei and yellow arrows show dead fibroblasts with clumping of cells. Observation of fibroblast morphology structure in the group; A. Comparison group; B. H_2O_2 only (negative control); C. Positive control; D. LgLE at 10 ppm; E. 20 ppm; F. 30 ppm; G. 40 ppm; H 50 ppm. Observation at 100X magnification.

Table IV - Proliferation rate of fibroblasts in 24 h, 48 h, dan 72 h of observations

Kelompok	Number of samples (N)	Proliferation (%) 24 h	48 h	72 h	*p value*
Fibroblast (comparison)	3	82.78 ± 3.86^{abc}	33.70 ± 1.27^{a}	15.38 ± 2.91^{ab}	0.00
Negative control	3	56.89 ± 11.20^{e}	19.05 ± 1.68^{b}	7.69 ± 4.79^{b}	
Positive control	3	86.45 ± 1.68^{a}	35.16 ± 2.20^{a}	12.82 ± 3.86^{ab}	
LgLE 10 ppm	3	85.71 ± 4.79^{ab}	35.90 ± 0.63^{a}	19.05 ± 2.29^{a}	
LgLE 20 ppm	3	72.53 ± 2.20^{bcd}	22.34 ± 4.58^{b}	10.26 ± 0.63^{b}	
LgLE 30 ppm	3	69.60 ± 0.64^{cde}	19.41 ± 5.64^{b}	9.52 ± 1.27^{b}	
LgLE 40 ppm	3	67.03 ± 2.91^{de}	19.78 ± 7.61^{b}	9.16 ± 1.68^{b}	
LgLE 50 ppm	3	66.30 ± 1.68^{de}	17.22 ± 0.63^{b}	7.33 ± 3.53^{b}	

$^{a-e}$ in various columns indicate significant differences ($p<0,05$)

to maintain the proliferation rate better rather than the comparison and positive control group. The microscopic observations of the fibroblasts' proliferation can be seen in Figure 4.

DISCUSSION

The extraction of lemongrass leaves using 70% ethanol solvent attracts more active compounds than other concentrations. According to Hasim et al. [11], lemongrass leaves that were extracted using a maceration process with 70% ethanol as a solvent had the highest antioxidant activity compared to 30% and 96% ethanol. Previous phytochemical tests on LgLE revealed that 70% ethanol includes active substances such as flavonoids, alkaloids, tannins, steroids, phenols, and saponins [24,25]. These active compounds had high antioxidant activity and free radical scavenging effects, which can be measured by the DPPH method [26]. If the IC_{50} value of a substance is less than 50 ppm, it is considered to have very high antioxidant activity, strong if it is 50-100 ppm, moderate if it is 100-150 ppm, and weak if it is 150-200 ppm. Based on the result in this study, it can be seen that the LgLE

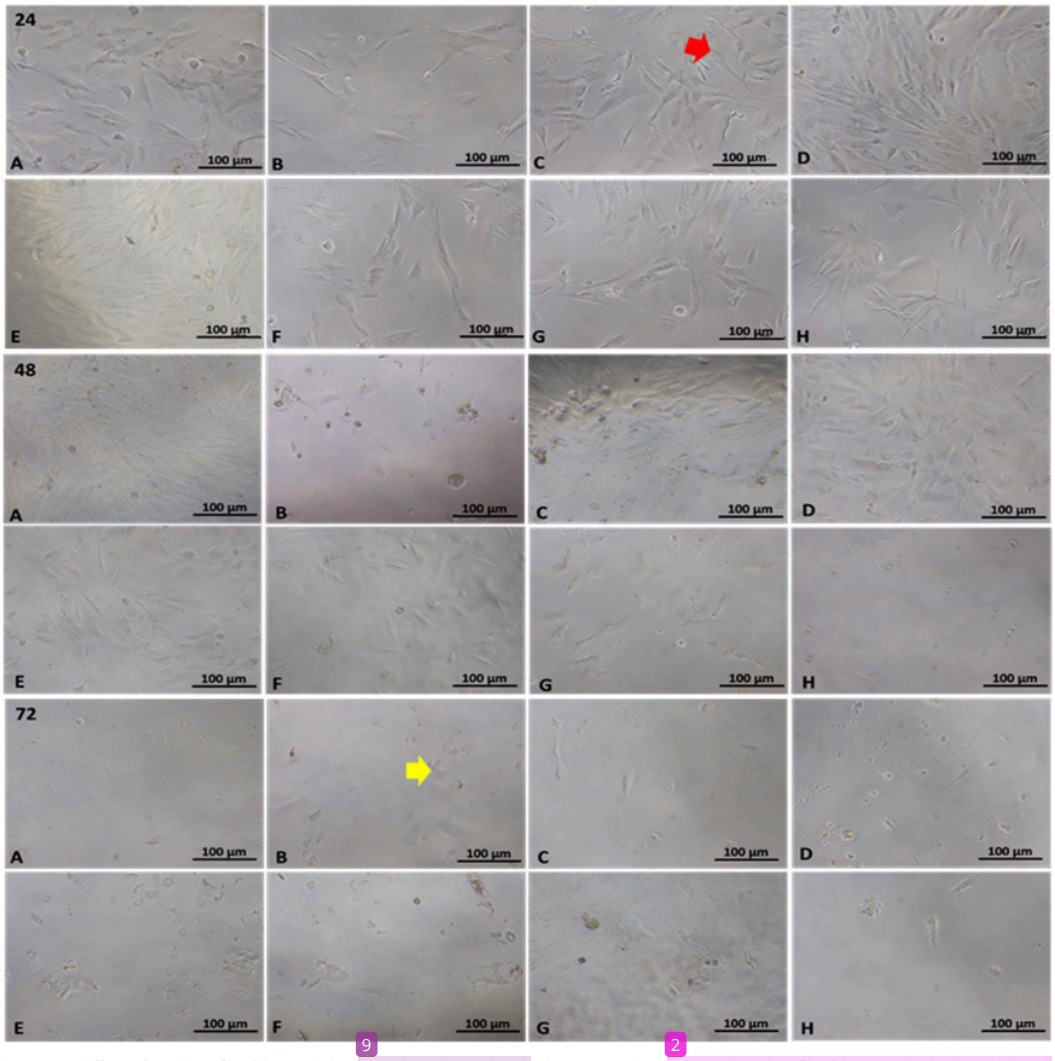

Figure 4 - Effect of LgLE on fibroblast viability at 24 h, 48 h, and 72 h observation. Red arrows show live fibroblasts with clearly visible cell nuclei and yellow arrows show dead fibroblasts with clumping of cells. Observation of fibroblast morphology structure in the group; A. Negative control; B. H_2O_2; C. Positive control; D. LgLE at 10 ppm; E. 20 ppm; F. 30 ppm; G. 40 ppm; H 50 ppm. Observations at magnification x100.

possessed a strong antioxidants activity [27]. This indicated that the active compounds contained in LgLE can neutralize the free radicals in DPPH which indicated by the change of colour in the DPPH method. The strong antioxidant activity was also due to the presence of secondary metabolic content such as flavonoids, phenolic acids, tannins, saponins, and alkaloids. In line with the other studies, LgLE possessed the same antioxidant activity with lemongrass extract [27] and citronella grass [28], with IC_{50} of 63,84 ppm and 58 ppm, respectively, which both are also categorized as a strong antioxidant.

To determine the oxidative stress of fibroblasts exposed by H_2O_2, the ROS assay was carried out, then LgLE in various concentrations were administered to examine their effect in order to attenuate ROS levels in cells that were experiencing an oxidative stress. Oxidative stress is characterized by the increase of ROS levels, which play an important physiological role during the wound healing process, such as chemical signaling of redox activity and activating phagocytic cells including neutrophils and macrophages, to attack the invasion of pathogens. Oxidative stress came about as a result of an unbalance antioxidant and pro-oxidant levels in cells, causing DNA, lipid, and protein damage inside cell membranes. This condition will later prolong the inflammatory phase of a chronic wounds because ROS will continue to stimulate chemotaxis as well as migration of neutrophils and macrophages to the wounded area. Furthermore, the direct cellular effects of excessive levels of ROS especially in wound healing process include the disruption of migration, proliferation, and synthesis of extracellular matrix by fibroblasts and keratinocytes. ROS also linked to the occurrence of several degenerative diseases such as alzheimers and parkinsons, and premature aging [29,30].

In this study results showed the LgLE can reduced the production of ROS which indicated by the lower percentage of green fluorescent cells compared to control. The LgLE contains secondary metabolics that can act as a non-enzymatic antioxidant which captured and neutralized free radicals, as well prevented the damage occured in fibroblasts. This non enzymatic antioxidant also donated its electrons, which cause free radicals became a more stable molecules and also breaking the chained reactions [31]. This result supports the findings of Veronica et al. [32], who reported that the antioxidant activity of lemongrass leaves might enhance the impact of chitosan microparticles, by lowering ROS generation in fibroblasts subjected to oxidative stress.

In the positive control group showed the lowest green fluorescence intensity, which indicates that the ascorbic acid can reduces the excessive production of ROS through several mechanisms including donating hydrogen atoms into lipid radicals, cleaving singlet oxygen, and converting tocoperoxyl radicals into alpha-tocopherol [33]. This is in line with the result of previous study where ascorbic acid possessed a good antioxidant, anti-inflammatory, and antiapoptotic effects, and also has a cytoprotective effect on ROS generated from the exposure of UVA and UVB radiation on fibroblasts and keratinocytes [34].

The potency of LgLE to sustain the survival of fibroblasts subjected to oxidative stress was demonstrated in ROS assay where it can reduce fibroblast oxidative stress. This may subsequently be observed in the increased value of live cells compared to the fibroblasts with only H_2O_2 induction and the untreated group, as proven by >80% of the surviving fibroblasts. The results are consistent with previous studies, which stated that LgLE maintains the viability of BHK-21 fibroblast cells because of the active compounds such as flavonoids and geraniol that have antioxidant effects on cells, and due to the high affinity of tannins which bind to proteolytic enzymes, and causes the enzymes fail to carry out their task of lysing cells, resulting in cells will remain viable [35].

The ability of LgLE in maintaining the viability of fibroblasts were also showed by its greater ability to induce the proliferation of fibroblasts at 24 h observation compared to those without treatment. With the help of secondary metabolites contain in lemongrass leaves, this extract can raise the number of fibroblasts that first suffer oxidative stress owing to an imbalance of oxidant and pro-oxidant levels, therefore lowering ROS levels in cells. According to Kaleci and Koyuturk [36], Moderate amounts of ROS can promote fibroblast growth in vitro, but at higher levels, they cause tissue damage and initiate a radical chain reaction.

The results revealed that administering LgLE at 10 ppm decreases oxidative stress on

fibroblasts and enhances viability, resulting in an increase in proliferation rates after 24 hours of observation, when compared to control groups. However, at greater doses, the effect was barely visible, as were viability and proliferation in LgLE groups. This is consistent with the findings of Maria et al. [31], who reported that synthesising silver nanoparticles with lemongrass leaf extract at a concentration of 10 ppm was the most efficient in lowering ROS generation in fibroblasts, and that concentrations beyond 10 ppm were less effective.

According to Jiang et al. [37], lemongrass essential oil eliminates excess free radicals and reduces cellular damage caused by oxidation in Human Embryonic Lung Fibroblasts, with the best visible effect at the lowest concentration and a decreased effect in maintaining cell viability at higher concentrations. Thus, 10 ppm of LgLE can maintain cells viability and accelarate the proliferation of fibroblasts under oxidative stress.

CONCLUSION

Conclusively, lemongrass leaves extract contains a secondary metabolic compound, which are then categorized as a strong antioxidant that can reduce the oxidative stress of fibroblasts conditioned during the healing of wounds, by balancing the ROS production, resulting in the increasing of the viability and growth of fibroblasts, with the most effective concentration is at 10 ppm.

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Author's Contributions

NF: Wrote the original draft, conducted the research and data collection, revise, read, and approved the submitted manuscript. DAB: Contributed to conception and design of the study, wrote, revised, read, and approved the submitted manuscript. K: Contributed to conception and design of the study, performed the statistical analysis, wrote, revised, read, and approved the submitted manuscript. IK: Contributed reagents and materials, performed the experiments, revise, read, and approved the submitted manuscript.

Conflict of Interest

The authors declare no conflict of interest.

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Regulatory Statement

The authors declare that this work does not require the approval of the ethics committee.

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