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Microencapsulation of Lemongrass Leaves Effect on Reactive Oxygen Species (ROS) Fibroblasts

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***Abstract*—Tissue damage during the inflammatory phase produces neutrophils and macrophages, which leads to excessive levels of reactive oxygen species (ROS). Excessive ROS level can cause progressive oxidative damage, thereby inhibiting wound healing. *Cymbopogon citratus* (lemongrass) leaves have active antioxidant compounds encapsulated with chitosan polymer, which improve the stability and effectiveness of active compounds in wound healing. This study examines thereduction of ROS production in fibroblasts under oxidative stress by encapsulating lemongrass (EChLgm). The study was divided into ten groups, untreated group, ascorbic acid, hydrogen peroxide, lemongrass extract and chitosan as controls, and five EChLmg concentrations of 100 ppm, 50 ppm, 25 ppm, 12.5 ppm and 6.5 ppm. The higher the EChLmg concentration, the more effective in reducing the ROS production on fibroblasts. The production of ROS on fibroblasts formed without the induction of H_2O_2 which was shown at a percentage of 50.62%. Ascorbic acid has the best ability to reduce ROS production with the intensity of 43.67%, while the stressor group produced the highest ROS with the intensity of 53.68%. The result showed that EChLmg group reduced ROS production on fibroblasts, with the best concentration of EChLmg at 100 ppm, with the intensity of ROS of 47.62%.**

***Keywords*—encapsulation, chitosan, lemongrass leaves extract, reactive oxygen species (ROS), fibroblast**

I. Introduction

A wound is a disturbance in the tissue in the form of continuity damage, caused by an injury or a surgical incision. In addition, it specifically refers to an injury that damages the epidermis of the tissues in the form of persistent pain. In dentistry, injury can result from physical or chemical trauma [1], and also iatrogenic agents during dental treatment. However, iatrogenic lesions can lead to the formation of traumatic ulcers, chemical or thermal burn [2]. The wound healing process occurs in three phases, namely inflammation, proliferation and remodeling [3].

The inflammatory phase generally lasts for several days and is characterized by homeostasis, chemotaxis, increased vascular permeability, wound closure, bacterial killing, and triggering cell migration [4]. In the proliferative phase, the wound matrix is replaced by granulation tissue made up of many fibroblasts, granulocytes, macrophages, blood vessels, and collagen, which restores the structure and function of the tissue [5]. In the remodeling phase, which is the final phase of wound healing, the granulation tissue formation ceases through cell apoptosis, followed by connective tissue reorganization and a contractile response [6].

In the inflammatory phase, neutrophils and macrophages invade the damaged area and produce ROS, reactive molecules derived from molecular oxygen and involved in various cellular functions, such as proliferation, apoptosis, migration, differentiation, and all critical events in the process of tissue homeostasis [7]. During the production of ATP by the endoplasmic reticulum or oxidoreductase, mitochondrial oxidative phosphorylation can also produce ROS [8]. The tissue damage can lead to excessive production of ROS, which result to progressive oxidative damage and ultimately to cell death [9], [10]. However, lemongrass leaves are natural ingredient that contain active compounds that can reduce the production of ROS, which accelerates wound healing.

Lemongrass is a plant widely used in tropical countries, especially in Southeast Asia, such as Indonesia [11]. Furthermore, it is a very important medicinal plant for wound healing and belongs to the Poaceae family. Lemongrass has bluish-green leaves that do not produce seeds and many rounded stems that increase in clump size as the plant grows. The roots are fibrous roots with short rhizomes [12]. Lemongrass uses are limited to the stem, and the leaves are often cast away [13]. The active compounds of lemongrass leaves contain antimicrobial, anti-inflammatory, antioxidant and increase fibroblasts proliferation, which play a role in wound healing [14].

The addition of antioxidants can inhibit the production of ROS and oxidative damage. Lemongrass leaves contain antioxidants such as flavonoids, alkaloids and tannins [15]. Antioxidants act as free radical scavengers by preventing and repairing the damage caused by ROS, increasing the body's defense capabilities, and reducing the risk of cancer and degenerative diseases [16]. The antioxidant content of lemongrass leaves is higher than that of the stems. Lemongrass leaves have high potential in a wide application in the health sector, especially tissue damage in the oral cavity due to trauma [17].

Active compounds from natural ingredients have limited bioavailability and stability, and this limitation can be controlled by encapsulating it in a matrix [18], such as chitosan [19], which is obtained from deacetylation of chitin, found in the shells of marine animals or insects [20]. In this study, chitosan from the exoskeleton of insects such as *Xylotrupes gideon* was used [21]. *X. gideon* is an insect that occurs frequently as a pest on coconut palms in Indonesia [22]. The use of chitosan to encapsulate extracts from other natural ingredients has been used, but *X. gideon* has never been used to encapsulate lemongrass. However, the physical modification to a smaller size aims to increase the effectiveness of encapsulation in reducing the production of ROS [20], [23]. The production of ROS can cause oxidative stress and disrupt the wound healing process, especially the proliferation of fibroblasts, which play a role in the synthesis of collagen proteins. Based on the existing problems, the purpose of this study was to determine the effect of chitosan encapsulation of lemongrass leaves ethanol extract on the production of ROS fibroblasts.

II. Materials and Methods

A. Chitosan Preparation

Chitosan is obtained from the beetle *X. gideon* from the Dramaga area, Bogor, West Java, Indonesia. The dentification of *X. gideon* was carried out at the Insect Museum of the Department of Plant Protection, Faculty of Agriculture, Bogor Agricultural University. Furthermore, *X. gideon* was separated into small pieces and dried for three days. Chitosan is made through demineralization (HCL 1N), deproteinization (NaOH 3N), and deacetylation (NaOH 50%) process, which were carried out at a ratio of 1:10 at 90°C for 1 hour. Chitosan was dried in an oven at 60°C for 5 hours. In this study, standard analytical solutions were used for laboratory procedures [24].

B. Lemongrass Leaves Extraction by Maceration Method

Lemongrass leaves were obtained from the Parung area, Bogor, West Java, Indonesia. The leaves were naturally dried for two weeks and then extracted using the maceration method. Furthermore, the leaves were soaked in 70% ethanol in a ratio of 1:10 at room temperature, then shaken manually for three days for 15 minutes every day, shaken repeatedly for 8 hours (3 times 24 hours). Then, the extract was filtered using Whatman filter paper and rotated using an evaporator at 50-60°C. Phytochemicals Lemongrass leaves were tested using the 18-16-2/MU/SMM-SIG (LCMS/MS) QTOF method (Saraswati Indo Genetech, Indonesia) [25].

C. Preparation of Chitosan Encapsulation of Lemongrass Leaves Extract

0.5 Grams of *X. gideon* chitosan was dissolved in 1% acetic acid, then 2 mL of 10% lemongrass leaves extract, and 100 mL of distilled water were added. Furthermore, the extract was stirred with a magnetic stirrer (IKA™ RH basic 2, Germany) at 2500 rpm for 2 hours. Subsequently, 40 mL of 0.1% tripolyphosphate was added dropwise and stirred for 1 hour. Then, 0.1 mL of tween 80 (0.1%) was added and stirred for 30 minutes. A Particle size test was performed on EChLmg using a Particle Size Analyzer (Malvern Zetasizer Nano series Nano-ZS) [26]. The process of lemongrass leaves encapsulation is shown in Fig. 1.

Fig. 1. The process of lemongrass leaves extraction by maceration method and encapsulation with chitosan by ionic gelation method.

D. ROS Calculation on Fibroblasts

2 The fibroblasts (BioCore Laboratory, Trisakti University) were grown in DMEM cell culture at 37°C and 5% CO_2. This study was divided into ten groups, namely the negative control group containing fibroblasts without EChLmg treatment (K0), the positive control group with ascorbic acid (K1), lemongrass leaves extract group (K2), chitosan (K3) and hydrogen peroxide (K4), with each concentration of 100 ppm, while the treatment group consists of EChLmg with concentrations of 100 ppm (P1), 50 ppm (P2), 25 ppm (P3), 12.5 ppm (P4), and 6.5 ppm (P5), incubated for 3 hours, then washed with 1X PBS. 1 mL of 2',7'-Dichlorodihydrofluorescein Diacetate (H2DCFDA) probe (Santacruz, USA) 10 M was added and incubated for 30 minutes. The groups were washed with 1X PBS and given 1 mL of 4',6-diamidino-2-phenylindole (DAPI) (Santacruz, USA) 300 M for 3 minutes. Then, the groups were washed using 1X PBS. Green fluorescent cells were observed using Zeiss Z1 Inverted Fluorescence (Germany) and counted using Image J software. The percentage of cells producing ROS are cells with green fluorescence intensity divided by the total number of cells multiplied by 100 [27].

III. Results and Discussion

The observation results of the active compounds in the lemongrass leaves extract as antioxidants are shown in Table 1.

This study confirmed the chitosan encapsulation effect of lemongrass leaves extract, which was physically modified into smaller particles. Chitosan is a cationic polymer that can react with multivalent anions such as tripolyphosphate. The ionic gelation method used in the encapsulation process of lemongrass leaves extract is a simple method with low toxicity and a slight possibility of changing the chemical properties of the encapsulated active compounds of lemongrass leaves extract. The chitosan encapsulation can extend the duration of active compounds or drugs, increase therapeutic efficiency, and reduce drug side effects because chitosan can open tight junctions on cell membranes and maintain ionic interactions, biocompatibility and biodegradability [28]. The characteristics of the EChLmg particle, based on the particle distribution, with a polydispersity index (PDI) of 0.603, indicates that the sample is heterogeneous or polydisperse [29].

The measurement of particle size distribution with the Malvern Zetasizer Nano series Nano-ZS shows that EChLmg has a dv90 of 6020 nm, which means that 90% of the total particle size of EChLmg is smaller than 6020 nm. The observation results showed that the average particle size of EChLmg is 1309 nm, and the particle size is microcrystalline. The observations result of the particle size distribution of EChLmg are shown in Table 2.

The average particle size of the encapsulated lemongrass leaves extract shows the results of physical modification using the ionic gelation method to produce microparticle size. Microparticles have a particle size of 1-100 μm. Microencapsulation can protect active compounds or drugs from the environment, cover unpleasant tastes, maintain the viability of surrounding cells, separate incompatible substances, protect from side effects, increase effectiveness, and extend the duration of active compounds [30].

TABLE I. PHYTOCHEMICAL RESULTS OF LEMONGRASS

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

TABLE II. ECHLMG PARTICLE SIZE DISTRIBUTION

	dv10 (nm)	*dv50 (nm)*	*dv90 (nm)*	*dv100 (nm)*	*Z-avg (nm)*
EChLmg particle	995	2590	6020	7460	1309

The results of the encapsulation of particles of chitosan lemongrass leaves extract showed a homogeneous or polydisperse size. The encapsulation or coating of active compounds from lemongrass leaves extract in microparticles is the most accessible manufacture particle with better effectiveness [31].

The results of the normality test with Shapiro-Wilk showed that the data had a normal distribution with $p>0.05$, continued by using the one-way ANOVA parametric test. The results of the One-way ANOVA test showed a significant difference ($p<0.05$) in the study group. Furthermore, the Tukey test was carried out. Other test results showed a significant difference between the untreated lemongrass leaves extract and the chitosan encapsulation group ($p<0.05$).

The group without EChLmg treatment (K0) and 50.62% of the green fluorescent cells showed a significant difference from the K1, K2, K4, P3, P2 and P1 groups ($p<0.05$). The results showed that the production of ROS on fibroblasts usually formed without the induction of stressors such as hydrogen peroxide. ROS is a natural product in normal cells involved in cellular signaling [32], and are free radicals produced in biological systems, such as oxidative phosphorylation and oxidation activity [33]. The increase in the ROS production due to the administration of hydrogen peroxide can induce oxidative stress and apoptosis in cells [34]. Oxidative stress is an imbalance between pro-oxidants and antioxidants, which plays a role in defense against tissue damage [33].

The K1 which received ascorbic acid as positive control showed a significant difference ($p<0.05$) from the other groups and the percentage of green fluorescent cells was the lowest, 43.67%. In the K2 which received the lemongrass leaves extract, the percentage of green fluorescent cells was 49.18%, showing a significant difference with groups K0, K4, and P5. However, for the K1 and P1 groups, the percentage of green fluorescent cells was significantly reduced compared to the K2 group. The percentage of green fluorescent cells in the K3 which received chitosan at 49.83%, slightly lower than that in the K2 group. Although the K2 and K3 groups were not significantly different from the K0 group, the two comparison groups showed a lower percentage of green fluorescent cells.

The group of fibroblasts administered by the stressor reduced the production of ROS after administration of lemongrass extract, although it was not as good as ascorbic acid. Lemongrass leaves extract contains active antioxidant compounds such as flavonoids, saponins, tannins, steroids, alkaloids, and triterpenoids. The antioxidant compounds in lemongrass leaves have the same antioxidant activity as other natural ingredients. The antioxidant activity of active compounds depends on the arrangement of functional groups based on the structure, configuration, and the total number of hydroxyl groups, which substantially affect the mechanism of antioxidant activity [35].

The administration of chitosan in fibroblasts exposed to oxidative stress reduced the production of ROS. Chitosan naturalizes free radicals through the binding of free radical ions such as hydrogen peroxide (H_2O_2), peroxide anions (O_2^-) and hydroxyl radicals (OH^-) with chitosan reactive groups, namely amino groups ($-NH_2$) and hydroxyl (-OH) [36]. The physical modification of chitosan into smaller particles can effectively regulate the body's main antioxidant enzymes such as superoxide dismutase, catalase and glutathione peroxidase. Chitosan regulates the activity of antioxidant enzymes and reduces lipid peroxidation [37].

The K4 group showed that the administration of hydrogen peroxide can cause oxidative stress in cells by increasing the production of ROS, which was observed in the highest green fluorescent cells by 53.68% compared to the other groups. The calculation of the number of green fluorescent cells in all groups is shown in Table 3.

Oxidative stress on fibroblasts can lead to fibroblast dysfunction. Fibroblasts are cells that play a role in the production of collagen and are responsible for tissue homeostasis. The presence of oxidative stress on fibroblasts stimulates collagen damage and the production of collagen itself [36]. Oxidative stress can reduce the production of ROS by providing antioxidants that can reduce damage caused by oxidative stress, thereby accelerating the wound healing process by reducing collagen damage and increasing the production of collagen, proliferation, migration, and secretion of growth factors [38], [39].

A group of fibroblasts with oxidative stress with H_2O_2 administration showed an increased the production of ROS, but with the administration of ascorbic acid, which acts as a non-enzymatic antioxidant to inhibit lipid peroxidation by completing the free radical electron deficiency and inhibiting the chain reaction of free radicals formation, can cause oxidative stress, thereby minimizing the occurrence of cell damage [40].

The EChLmg treatment group showed that the group that received an EChLmg concentration of 100 ppm (P1) was not significantly different at P=1,000 from the EChLmg P2 group. However, compared with the P2 group, the P1 group has a lower percentage of green fluorescent cells compared to the P2 group. The P1 group with the lowest rate of green fluorescent cells at 47.62% showed significant differences with the P3 ($p=0.476$), P4 ($p=0.04$), and P5 ($p=0.032$) groups. The P2 group, which received 50 ppm EChLmg, had a green fluorescence cell intensity of 48.48%, lower than the P3 group of 49.29%. The group that received EChLmg concentration of 12.5 ppm (P4) showed the p-value = 0.991, and the percentage of green fluorescent cells is 50.14%. An EChLmg concentration of 6.5 ppm (P5) showed 51.21% green fluorescent cells. Although there was no significant difference, the green fluorescent cells in the P5 group were slightly higher than those in the P4 group. The P4 group showed a p value= 0.319 with the P3 group, but the percentage of green fluorescent cells in the P3 group was slightly lower than the P4 group.

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

Groups	*Number of Samples (n)*	*Green Fluorescent Intensity (%)*	*P-value*
Fibroblasts	3	50.62 ± 0.97^{hc}	0.000
Ascorbic Acid	3	43.67 ± 1.39^{g}	
Lemongrass Leaves	3	49.18 ± 0.59^{de}	
Hydrogen Peroxide	3	53.68 ± 0.37^{a}	
Nano Chitosan	3	49.83 ± 0.36^{cd}	
EChLmg 6.5 ppm	3	51.21 ± 0.33^{b}	
EChLmg 12.5 ppm	3	50.14 ± 0.19^{bcd}	
EChLmg 25 ppm	3	49.29 ± 0.76^{de}	
EChLmg 50 ppm	3	$48,48 \pm 0.96^{ef}$	
EChLmg 100 ppm	3	47.62 ± 0.08^{f}	

*a-f In different columns showed significant differences (p<0.05)

Encapsulation of chitosan in lemongrass leaves can reduce the production of ROS on fibroblasts, with higher concentrations of EChLmg. Furthermore, encapsulation aims to protect the active compounds of natural ingredients from decomposition and control their release [41]. The results showed that the higher the encapsulated concentration of chitosan, lemongrass leaves extract, the more effectively the production of ROS was reduced. Chitosan which encapsulates the active compounds of lemongrass leaves can work on its own. However, the active antioxidant compounds in lemongrass leaves can increase the effect of chitosan microparticles, and therefore reducing ROS production, which is in line with another study where gelatin B and chitosan were used to encapsulate curcumin. That study has shown that the microencapsulation of curcumin can improve the integrity and stability of curcumin's antioxidant components [19].

The results of the EChLmg treatment at various concentrations showed that the higher the stated concentration, the lower the production of ROS produced by fibroblasts. The intensity of green fluorescent cells can be used to observe the production of ROS fibroblasts with the intensity of the green fluorescent cells, while the intensity of blue fluorescent can be used to observe cell nuclei stained by a DAPI probe. Subsequently, when compared with other EChLmg groups, the group that received 100 ppm EChLmg has an excellent ability to reduce ROS production and lower the intensity of green fluorescent cells. The group that received the stressor showed the highest production of ROS compared to the other groups, and the group that received ascorbic acid inhibited the production of ROS and had the lowest intensity of green fluorescent cells compared with other groups. Furthermore, the lemongrass leaves extract group and chitosan group slightly decreased the production of ROS. However, the encapsulation of lemongrass leaves extracts with chitosan in microparticles can reduce the production of ROS, clearly visible in several concentration groups such as P3, P2 and P1 groups. An illustration of the intensity of green and blue fluorescent cells in all study groups is shown in Fig. 2.

Lemongrass leaves extract contains active compounds with antioxidant activity that can reduce the production of ROS. The encapsulation showed high interactivity of the active compound with chitosan polymer in order to protect from oxidation and increasing its therapeutic potential [42].

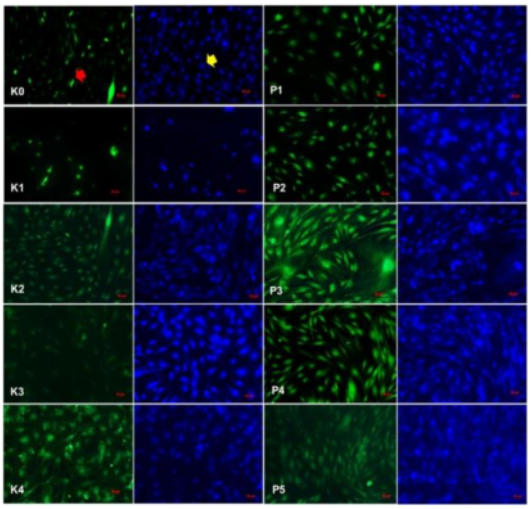

Fig. 2. The production of ROS was measured on fibroblast with the H2DCFD-DA probe (green) and the DAPI probe (blue). The red arrow shows ROS fibroblasts with green fluorescence intensity, and the yellow arrow shows fibroblast nuclei with blue intensity. The green fluorescence intensity in the untreated group (K0), the ascorbic acid group (K1), the lemongrass leaves extract group (K2), the chitosan group (K3), and the hydrogen peroxide group (K4). The group received EChLmg at concentrations of 100 ppm (P1), 50 ppm (P2), 25 ppm (P3), 12.5 ppm (P4) and 6.5 ppm (P5). Observations were carried out at 100X magnification.

IV. CONCLUSIONS

According to the study results, the encapsulation of lemongrass leaves extracts with physically modified chitosan produced microparticles that effectively reduced the production of Reactive Oxygen Species (ROS), with a low intensity of fibroblast green fluorescence cells. In addition, the higher concentration of EChLmg the more effective in reducing the production of ROS on fibroblast, which was visible at a concentration of 100 ppm with a ROS intensity of 47.62%.

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