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NANO ENCAPSULATION of LEMONGRASS LEAVES EXTRACT (*Cymbopogon citratus* DC) on FIBROBLAST VIABILITY with OXIDATIVE STRESS

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ABSTRACT

Background(s):

During the wound healing process, fibroblasts may experience oxidative stress due to the high number of *Reactive Oxygen Species* (ROS) produced by inflammatory cells, which, if out of control, may cause impaired fibroblast function, leading to a slow wound healing process. Non-enzymatic antioxidants as suppressors for oxidative stress are derived from secondary metabolites of natural ingredients such as lemongrass, which is unstable when reacting with the external environment and can be minimised by encapsulation using a natural polymer of chitosan

Objective(s):

The purpose of this research is to find out the antioxidant activity of chitosan encapsulated lemongrass leaves extract (EnChLg) and its effect on the viability of fibroblasts under oxidative stress.

Methods:

Antioxidant activity was measured using the 2,2-diphenyl-2-picrylhydrazil (DPPH) way. Viability and proliferation tests were conducted using the Cell Counting Kit-8 (CCK-8) at a wavelength of 450 nm. The research was divided into 8 groups consisting of groups with hydrogen peroxide, ascorbic acid, groups without treatment and EnChLg treatment with concentrations of 100 ppm, 200 ppm, 300 ppm, 400 ppm, 500 ppm.

Result(s):

The EnChLg antioxidant activity test showed antioxidant activity with an IC_{50} value of 566.48 ppm, and administration of EnChLg with a concentration of 300 ppm was able to

increase the viability of fibroblasts better than the concentrations of 100 ppm, 200 ppm, 400 ppm, and 500 ppm as well as the positive, negative and comparison control groups.

Conclusion(s):

EnChLg has weak antioxidant activity. However, EnChLg is non-toxic and fibroblasts were able to survive well at a concentration of 300 ppm.

Keywords:

Lemongrass, encapsulation, chitosan, oxidative stress, fibroblasts

BACKGROUND(s)

Wound healing is the body's main defense mechanism through several phases, hemostasis, inflammation, proliferation, and remodeling.[1] The hemostasis is the initial phase characterized by the vasoconstriction of blood vessels and coagulation,[2] aiming at preventing excessive blood loss[3] and maintaining tissue integrity.[4] In the inflammatory phase, phagocytic cells, such as neutrophils and macrophages, will move to the injured area and experience a respiratory burst,[5] namely an increase in the use of oxygen, and the Reactive Oxygen Species (ROS) will be produced,[6] such as superoxide and hydrogen peroxide, which act as bactericidal.[7] The low amount of ROS has beneficial effects on several physiological processes of the body, such as the wound healing process. However, too high levels of ROS can increase pro-oxidants and lead cells to experience oxidative stress.[8] Oxidative stress is a condition when there is an overproduction of ROS and a deficiency of enzymatic antioxidants, causing an imbalance between the number of pro-oxidants and antioxidants in cells.[9] This imbalance can interfere with the function of communication between cells and cause tissue damage, thereby disrupting the wound healing process, such as prolonging the inflammatory phase and inhibiting the proliferation process and collagen formation by fibroblasts.[10]

Herbal medicines have been widely used in the wound healing process. One of the herbal medicines is *Cymbopogon citratus* DC, known as lemongrass. Lemongrass is one of the spices growing in the tropics and is widely used in Southeast Asia, such as Indonesia.[11] Based on scientific studies, the lemongrass leaf has a higher content of active compounds than its stem,[12] The active compounds contained in lemongrass have antioxidant activities, such as flavonoids, tannins, saponins, alkaloids, and phenols.[13] Natural ingredients with antioxidants have been proven to accelerate the wound healing process.[14] One way to use the natural ingredients with antioxidants is by reducing the production of reactive oxygen species (ROS),[15] which can cause oxidative stress to cells, thereby slowing the wound healing process.[16]

The active compounds in the body have limited bioavailability and absorption in the body, which can be controlled by encapsulation technology. Encapsulation is the trapping of active compounds to enhance bioavailability and stability,[17] such as the protection of active compounds against oxidation to upgrade their therapeutic potential.[18] The polymer material commonly used as a trapping matrix is chitosan.[19] Chitosan is a natural polymer compound obtained from chitin in the shells of marine animals and insects,[13] such as the horned beetle (*Xylotrupes gideon*) which is a pest on coconut plants but it has economic value by converting it into natural ingredients, such as chitosan.[13] Chitosan is widely used due to its potential to be developed as a drug delivery since it is biocompatible, biodegradable, has a low level of toxicity, and is a simple preparation method.[20] Physical modification of chitosan into smaller particles can increase absorption, diffusion, and penetration to the mucosal layer better than chitosan with regular size.[21]

Based on the description in the background, this study aimed to determine the antioxidant activity of nano-chitosan encapsulation of lemongrass leaf extract (EnChLg) on the viability of fibroblasts subjected to oxidative stress due to hydrogen peroxide induction.

METHODS

Lemongrass Leaf Extraction

Leaves of *C. citratus* and *X. gideon* were obtained from Bogor, West Java, Indonesia. The making process of chitosan from *X. gideon* was carried out in several stages, namely demineralization, deproteinization, decolorization, and deacetylation. The lemongrass leaves were dried for two weeks naturally. Extraction was conducted by maceration method using 70% ethanol solvent (1:10 w/v),[22] for 72 hours at room temperature.[23] Then, manual shaking was performed for three days. Shaking was executed every day for 15 minutes and repeated for eight hours. After three days, the extract was filtered using Whatman filter paper and evaporated using a rotary evaporator at a temperature of 50-60^{o}C to concentrate the filtrate until a thick extract was obtained.[24] The study was divided into eight

groups, namely fibroblasts without treatment as a negative control, fibroblasts given ascorbic acid as a positive control, fibroblasts given a stressor, and fibroblasts given EnChLg treatment with concentrations of 100, 200, 300, 400, and 500 ppm.

Nano-Chitosan Encapsulation of Lemongrass Extract

Nano-chitosan encapsulation was conducted by dissolving 0.5 grams of chitosan with 1% of acetic acid. One gram of lemongrass leaf extract was weighed and then dissolved with 10 mL of distilled water. A total of 150 mL of extract solution was mixed into 300 mL of chitosan solution, then stirred using a magnetic stirrer at a speed of 2500 rpm for 20 minutes. After that, the stirring was continued without heating for 100 minutes, then the solution was added with 40 mL of tripolyphosphate 0.1% dropwise. Then, the solution was stirred for one hour at a speed of 2500 rpm. After that, the tween 80 solution with 0.1% concentration was added and stirred again at 2500 rpm for one hour.[12] The samples were centrifuged again at 7100 rpm for 15 minutes to obtain a precipitate, then dilution was carried out at concentrations of 100, 200, 300, 400, and 500 ppm.

Antioxidant Activity of EnChLg

The antioxidant activity test of EnChLg was carried out using the 2,2-diphenyl-1-picrylhydrazyl method. The samples of EnChLg were prepared from 10,000 ppm stock solution and diluted with methanol. The DPPH solution was prepared by dissolving 20.2 mg of it in 126.25 mL of methanol in a volumetric flask. The extract solution was taken using a pipette (Eppendorf) and put into a test tube, then added with DPPH solution and methanol so that the volume of the solution reached 5 mL. The solution was shaken with a vortex mixer (Thermo Scientific) until homogeneous and then transferred to a test tube covered with aluminum foil. Subsequently, the solution was incubated for 30 minutes in a dark room. After that, the absorbance was measured at a wavelength of 516 nm.[25] The percentage of inhibition can be calculated by the following formula:[26]

$$\text{Inhibition}(\%) = \frac{\text{Blank Absorbance} - \text{Sample Absorbance}}{\text{Blank Absorbance}} \times 100$$

The IC50 calculation is determined from the results of the linear regression equation y = a + bx from various sample concentrations where the x-axis is the sample concentration, the y axis is the percentage of inhibition, a is the intercept or the intersection of the y line and b is the slope. Then, IC50 can be calculated using the following formula:[27]

$$\frac{50 - a}{b}$$

Viability Test

Fibroblasts previously frozen in liquid nitrogen were warmed at 37°C in a water bath until thawed. After that, the fibroblasts were centrifuged at 1500 rpm for 15 minutes to obtain cell pellets.[28] The precipitate from the centrifugation was stored in a laminar flow. Then, the cells were suspended again on a culture medium scaffold made with DMEM (Gibco), 10% FBS, and 1% antimycotic antibiotic in the form of penicillin-streptomycin.[29] Then, the cells were grown with expansion using T-flask and incubated for 48 hours at 37ºC. The cultured cells were divided into eight groups. The fibroblast culture in the group expected to experience oxidative stress was added with a stressor in the form of 100 M H_2O_2 and then stored in a 5% CO_2 (ESCO) incubator at 37ºC for 60 minutes.[30]

Fibroblasts were planted on 96 well plates of 10,000 cells/well on culture media which had been replaced with 200 L centrifuged sample solution. Then, the fibroblasts were incubated for 24 hours. After being treated and incubated, the cells were washed using PBS 10% once. Then, 100 L of CCK-8 (Dojindo) reagent was added to each well. The wells were incubated at 37ºC for 90 minutes. Viability and cytotoxicity tests were observed after 24 hours.[31]

$$\text{Percentage of cell viability (\%)} = \frac{\text{Absorbance A} - \text{Blank Absorbance}}{\text{Absorbance B} - \text{Blank Absorbance}} \times 100$$

RESULT(s)

This study was conducted to determine the effect of lemongrass leaf extract entangled with a natural polymer in the form of horn beetle chitosan in particle size modified into nanoparticles to increase stability, solubility, and its effectiveness in influencing the activity of fibroblasts subjected to oxidative stress. The fibroblasts used in this study were Human Dermal Fibroblasts (HDF) from the biorepository of Yarsi University. The lemongrass used was identified by the Biological Research Center of the Biological Sciences Organization of West Java No. B-112/V/DI.05.07/9/2021.

In this study, the effect of nano-chitosan encapsulation of lemongrass leaf extract (EnChLg) on the activity of fibroblasts under oxidative stress was examined. Oxidative stress of fibroblasts was obtained by giving H_2O_2 solution and incubated for 60 minutes indicated by the results of oxidative stress observations, namely the formation of reactive oxygen species (ROS) from fibroblasts.

Antioxidant Test of EnChLg

The antioxidant activity of EnChLg was determined by performing the 2,2-diphenyl-1-picrylhydrazyl (DPPH) test. The DPPH test is a simple, easy, fast, reactive test method, and it does not require many samples to determine the antioxidant activity of a compound. Antioxidant activity can be identified by quantitative measurement of a compound in capturing free radicals from DPPH using UV-Vis spectrophotometry for absorbance measurement so that the value of the antioxidant activity of the compound is attained by reducing free radicals expressed by the IC_{50} value. The IC_{50} value is the concentration of a compound capable of reducing free radicals up to 50%.[33]

The results of the EnChLg antioxidant test with concentrations of 100, 200, 300, 400, and 500 ppm showed that the mean percentage of EnChLg inhibition was 10.48%, 19.16%, 25.89%, 36.71, and 31.42%, respectively as can be seen in Table 1.

Observation of the inhibition percentage results can determine the IC_{50} value. The IC_{50} value of chitosan encapsulation of lemongrass leaf extract was 566.48 ppm. The calculation of the IC_{50} value can be obtained from the calculation results of the linear regression equation, the regression equation

with the values of y = 0.078x + 4.1972 and R^2 = 0.8712, y = 0.0757 x+5.2709 and R^2 = 0.8847, and y = 0.0915x+ 2.2976 and R^2 = 0.9924 from the first, second, and third EnChLg tests respectively. The results of the regression equation from EnChLg in three replications can be seen in Figure 1.

Cytotoxicity of EnChLg

A cytotoxicity test was conducted to determine the value of Inhibitor Concentration (IC_{50}), which is the concentration of compounds capable of inhibiting cell growth up to 50%. The smaller the IC_{50} value produced, the compound will be more toxic. Cytotoxicity test of EnChLg on fibroblasts subjected to oxidative stress showed an IC_{50} result of 369.33 ppm. The calculation of the inhibition percentage in determining the IC_{50} value was obtained from the regression equation with the value of y = 0.1043x + 11.479 and R^2 = 0.9351 (Figure 2).

Viability of EnChLg

Based on the results of observations and readings of the absorbance values of the viability test from EnChLg concentrations of 100, 200, 300, 400, and 500 ppm on fibroblasts, the results of the normality test using Shapiro-Wilk showed that the data had a normal distribution with p>0.05. The test was continued with the Oneway ANOVA parametric test demonstrating that there was a significant difference in the study group (p<0.05). Next, Duncan's test was conducted to find out which groups had differences. The results of Duncan's test indicated that there were significant differences in the untreated group and the encapsulated group of lemongrass leaf extract with chitosan.

The viability test results showed that the group without treatment as negative control did not indicate a significant difference (p>0.05) from the positive control group given ascorbic acid and the group given EnChLg with a concentration of 300 ppm with viability values of 87.67%, 87.67%, and 90.00%, respectively. However, the EnChLg concentration of 300 ppm had a higher percentage of 2.66% compared to the negative and positive control groups. The negative control group, positive control group, and EnChLg had 300 ppm significant differences from the group given the H_2O_2 stressor, EnChLg 100 and 500 ppm with viability values of 68.33%, 70.50%, and 71.33%,

respectively. The group that was given stress, although it did not show a significant difference, it had the lowest viability value compared to the EnChLg 100 and 500 ppm groups, namely 3.18% and 4.39%, respectively. Although the EnChLg 400 ppm group was not significantly different from the other groups, it had higher viability than the group given the stressor, EnChLg 100 and 500 ppm. Meanwhile, EnChLg 400 ppm had low viability when compared to the EnChLg 200 and 300 ppm, positive and negative control groups. The calculation results of the viability values can be seen in Table 2. The results of microscopic viability test observations in all study groups showed the persisted fibroblasts and fibroblasts undergoing apoptosis. The results of microscopic observations at 100x magnification can be seen in Figure 3.

DISCUSSION

The success of the wound healing process is supported by mutually coordinated and overlapping healing phases, namely hemostasis, inflammation, proliferation, and remodeling, as well as various mediators, growth factors, and cells involved in this process, one of which is fibroblasts. Fibroblasts are cells of which populations are mostly found in connective tissue and are one of the cells playing a significant role in the wound healing process, especially in the proliferative phase. Fibroblasts are stimulated by various mediators and growth factors to proliferate and migrate to the wound area which then plays a role in depositing collagen fiber proteins and the ground substance forming an extracellular matrix (ECM) to facilitate cell migration and form new tissue.[34]

In the wound healing process, the role of oxygen (O_2) is required to produce adenosine triphosphate (ATP) by the mitochondria, thereby increasing the energy needed for the formation of new tissue. However, O_2 molecules are having unpaired electrons so they become very reactive molecules, namely Reactive Oxygen Species (ROS). The most common forms of ROS are superoxide (O_2-), hydrogen peroxide (H_2O_2), and hydroxyl (OH-) anions. Too low levels of ROS in the body can induce a halt in the cell cycle, whereas normal levels of ROS can maintain cell function, and

excessive levels of ROS can result in the activation of pro-apoptotic proteins causing the death of cells.[35] Therefore, the amount of ROS in the body needs to be balanced with the presence of antioxidants that will work synergistically in balancing ROS in the inflammatory process.[36]

Ascorbic acid is a non-enzymatic antioxidant from natural ingredients that have been known as the gold standard of antioxidants. Ascorbic acid can balance the amount of ROS by neutralizing superoxide anions and hydroxyl radicals and activating other antioxidants in the body.[36] Apart from ascorbic acid, other natural antioxidants are obtained from active compounds in almost all plants, one of which is lemongrass leaf or *Cymbopogon citratus*. Extracts from lemongrass leaves are proven to contain active compounds of flavonoids, phenols, tannins, saponins, and alkaloids known as natural non-enzymatic antioxidants.[37] To enhance the stability and protect the active compounds from the extract of lemongrass leaf, trapping is carried out with natural polymers in the form of chitosan,[38] physically modified into nanoparticles. The chitosan used is derived from the horned beetle or *Xylotrupes gideon* which is a plant pest but it has beneficial properties, such as biocompatible, biodegradable, mucoadhesive, low toxic, and it has good diffusion and penetration capabilities.[13]

The DPPH value indicates the antioxidant activity of a compound with the magnitude of the free radical scavenging activity of DPPH indicated by the IC_{50} value. The smaller the IC_{50} value produced by a compound, the antioxidant activity will be higher.[39] The DPPH test results showed that EnChLg has antioxidant activity with a strong low IC_{50}. This is based on the classification of IC_{50} values of 10-50 ppm containing strong antioxidant activity, 50-100 ppm containing moderate antioxidant activity, and IC_{50} of more than 100 ppm containing strongly low antioxidant activity.[40] The antioxidant activity of EnChLg occurs when the EnChLg active compound donates a hydrogen atom to an unpaired electron in the free radical strand indicated by a change in the color of DPPH from purple to yellow.[41] Hence, the active compound of lemongrass leaf extract ensnared with nano chitosan can be a natural alternative to antioxidants to help balance the number of free radical molecules with a better delivery and absorption system in the body. This is following research conducted by Husniati,[42] revealing that the antioxidant activity of the anthocyanin extract of *Euphorbia milii* flower encapsulated with

chitosan could be maintained for good storage and delivery. Also, research by Sari et al.,[43] identified that lemongrass leaf extract had antioxidant activity as a free radical scavenger and secondary antioxidant since it could reduce redox potential and stabilize the oxidized form of metal ions.[43]

The antioxidant activity with an extremely IC_{50} from EnChLg indicated a cytotoxicity value with a non-toxic IC_{50}, where an active compound is considered to be toxic if it has an IC_{50} category value of less than 1000 ppm.[44] This is shown by the viability value of fibroblasts given EnChLg 300 ppm could survive up to 90%. The high cell viability value showed that EnChLg administration could not only reduce oxidative stress due to hydrogen peroxide administration (stressor), but EnChLg administration could escalate fibroblast viability as indicated by a higher viability value than the negative control. Furthermore, the EnChLg administration had better viability than the positive control given a synthetic non-enzymatic antioxidant in the form of ascorbic acid. In this study, observations of fibroblast viability showed that the viability value of the EnChLg treatment indicated a higher number than the control, especially at a concentration of 300 ppm giving the highest viability value. This is in line with research carried out by Sari et al.,[43] revealing that the ethanolic extract of lemongrass leaves showed capable antioxidant activity in scavenging hydrogen peroxide and hydroxyl, where hydroxyl can cause a direct effect on lipid peroxidation and hydroxyl is the most damaging ROS for cell components.

Research undertaken by Pan et al.,[45] found that the antioxidant activity could enhance cell viability and proliferation by reducing oxidative stress caused by high amounts of ROS and lipid peroxidation. This is following research conducted by Roriz et al.,[46] identifying that lemongrass could reduce lipid peroxidation in brain cells due to the high concentration of flavonoids contained in the brain cells. Moreover, lemongrass exhibits an antioxidant effect by increasing the activity of the enzyme superoxide dismutase (SOD) and decreasing the production of ROS in macrophages.[108] In the wound healing process, by decreasing oxidative stress and ROS production in cells, inflammatory cells will be stimulated to release various mediators and growth factors, such as interleukin-1β, platelet-derived

growth factor (PDGF), and basic fibroblast growth factor (BFGF) which will stimulate the proliferation of fibroblasts.

CONCLUSION(s)

Nano chitosan encapsulation of lemongrass leaf extract contains active compounds playing a role as antioxidants effective in accelerating the wound healing process by reducing the level of oxidative stress from fibroblasts, through high viability. The concentration of EnChLg having the best and most significant effect on fibroblast viability was the concentration of 300 ppm.

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CONFLICT OF INTEREST

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TABLES

Table 1. Mean Percentage of Inhibition and IC_{50}

EnChLg	Inhibition (%)			Mean	IC_{50} (ppm)
	1	2	3		
100	7.68	11.51	12.25	10.48 ± 2.45	
200	20.53	17.16	19.80	19.16 ± 1.77	
300	34.86	34.76	30.20	25.89 ± 2.67	566.48 ± 31.95
400	35.89	37.13	37.09	36.71 ± 0.70	
500	38.99	39.39	49.34	31.42 ± 5.87	

Table 2. Fibroblast viability mean

Group	Number (N)	Viability ± SD (%)	*p Value*
Fibroblast	3	87.67 ± 6.51[a]	
H_20_2	3	68.33 ± 7.64[c]	
Ascorbic acid	3	87.67 ± 1.53[a]	
EnChLg 100	3	70.50 ± 5.50[bc]	
EnChLg 200	3	85.00 ± 6.56[ab]	$p<0.05$
EnChLg 300	3	90.00 ± 4.36[a]	
EnChLg 400	3	81.67 ± 11.59[abc]	
EnChLg 500	3	71.33 ± 13.50[bc]	

[a-c] the different columns show significant differences ($p<0.05$)

FIGURES

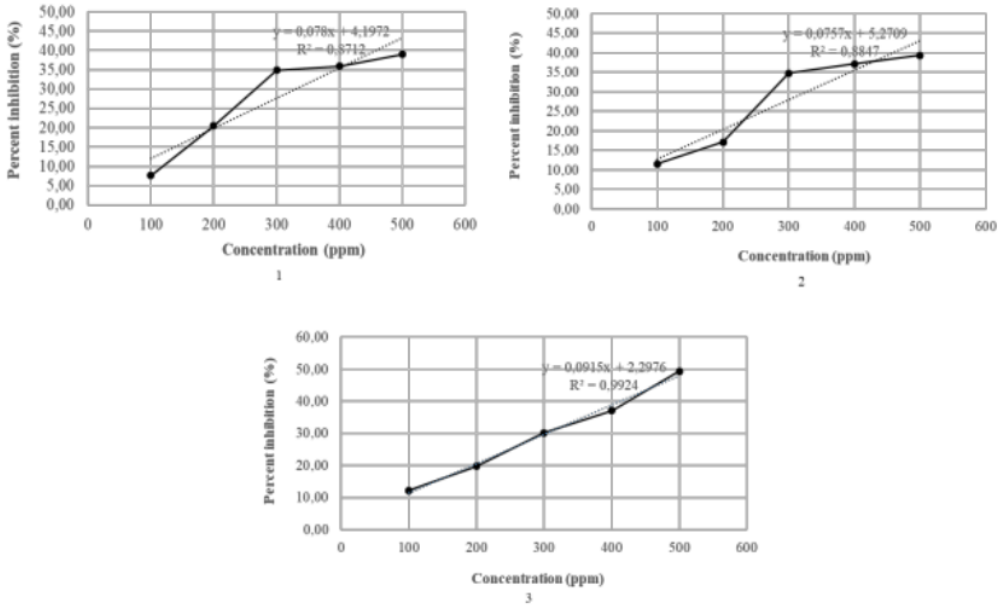

Figure 1. Curve of the relationship between the EnChLg concentration and the inhibition percentage

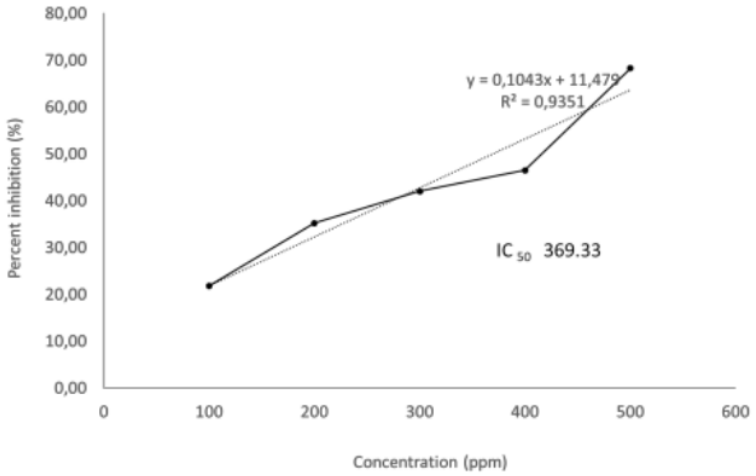

Figure 2. IC_{50} Value of Cytotoxicity of EnChLg

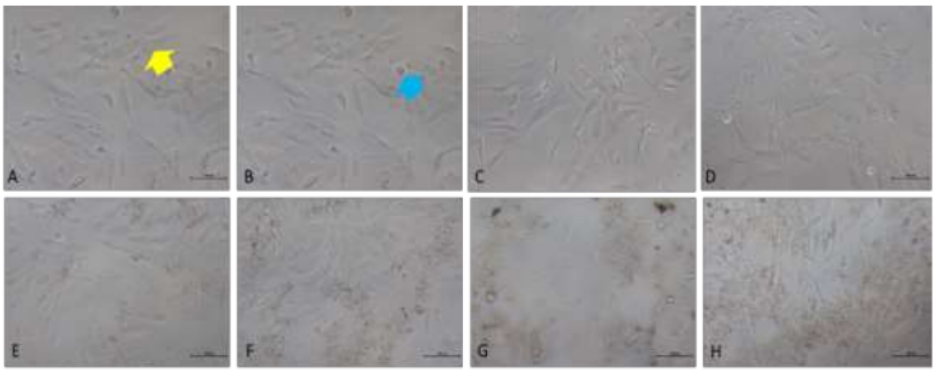

Figure 3. Effect of EnChLg administration on fibroblast viability. Yellow arrows show live fibroblasts with clearly visible cell nuclei and blue arrows indicate dead fibroblasts with clumping of cells. Observation of fibroblast morphology structure in the groups: A. Negative control, B. H_2O_2, C. Positive control, D. EnChLg 100 ppm, E. EnChLg 200 ppm, F. EnChLg 300 ppm, G. EnChLg 400 ppm, H. EnChLg 500 ppm. Observations were at 100x magnification.

NANO ENCAPSULATION of LEMONGRASS LEAVES EXTRACT (Cymbopogon citratus DC) on FIBROBLAST VIABILITY with OXIDATIVE STRESS

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