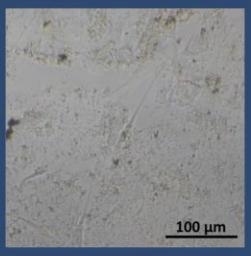
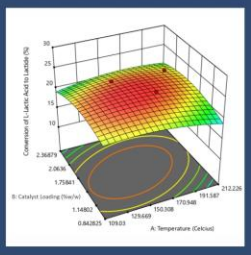
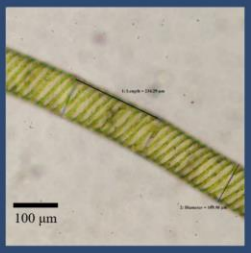
Indonesian Journal of Biotechnology

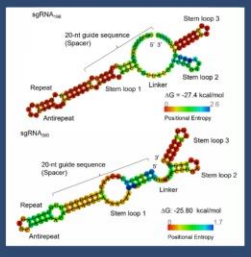
Vol. 28, No. 4, December 2023 ISSN 0853-8654 (Print) | ISSN 2089-2241 (Online)

ARTICLES









Indonesian Journal of Biotechnology

COUNTRY	SUBJECT AREA AND CATEGORY	PUBLISHER	SJR 2024
Indonesia Universities and research institutions in Indonesia Media Ranking in Indonesia	Agricultural and Biological Sciences Agricultural and Biological Sciences (miscellaneous) Food Science Biochemistry, Genetics and Molecular Biology Biotechnology Environmental Science Environmental Science (miscellaneous)	Universitas Gadjah Mada, Research Center for Biotechnology	0.191 Q3 H-INDEX 7

PUBLICATION TYPE	ISSN	COVERAGE	INFORMATION
Journals	08538654, 20892241	2018-2024	Homepage

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VOLUME 28(4), 2023, 191-199 | RESEARCH ARTICLE

Chitosan *Xylotrupes gideon* encapsulated lemongrass leaf ethanol extract reduce H_2O_2-induced oxidative stress in human dermal fibroblast

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SUBMITTED 20 January 2023 **REVISED** 4 July 2023 **ACCEPTED** 15 August 2023

ABSTRACT During phagocytosis, phagocyte cells discharge reactive oxygen species referred to as respiratory bursts, inducing a rise in pro-oxidants and subjecting the cell to oxidative stress. Such stress is a biological mechanism related to an imbalance in pro-oxidant/antioxidant homeostasis, which generates toxic reactive oxygen. Encapsulation is a coating process to improve the stability of bioactive compounds from lemongrass extract. Therefore, this study aims to determine the encapsulation activity of lemongrass leaf extract with chitosan *X. gideon* (LEChXg) to reduce the oxidative stress of fibroblasts. The research used the human dermal fibroblast (HDF) cell line, comprising negative and positive controls and use of LEChXg 100, 200, 300, 400, and 500 µg/mL. HDF cell migration was evaluated by employing the scratch wound healing method and the wound closure was oberseved at 0, 2, 4, 6, and 24 h intervals. The cell proliferation was observed at 24, 48, and 72 h using CCK-8 at a 450 nm wavelength. The results showed that the observations at 0, 2, and 4 h did not demonstrate any significant difference on the cell migration ($p > 0.05$) among the groups. However, the wound closure at 4 and 6 h showed a significant difference ($p < 0.05$) with LEChXg 300 µg/mL. Despite the lack of any significant variation observed up to 24 h, fibroblast subjected to the stressor did not achieve complete closure. The groups treated with LEChXg were more stable in maintaining fibroblast proliferation up to the end of the observation than those with stressors at 24, 48, and 72 h. Fibroblast induced with a stressor was also more stable in maintaining migration and proliferation in groups receiving LEChXg 300 µg/mL.

KEYWORDS Chitosan *X. gideon*; Lemongrass leaf; Migration; Oxidative stress; Proliferation

1. Introduction

Mouth or oral ulcers are generally known as discontinuities of oral mucosa characterized by epithelial tissue damage and connective tissue in lamina propria of the mucosa in oral cavity (Zakiawati et al. 2020). About 40% of people have been estimated to suffer from oral ulcers disease (Zakiawati et al. 2020) due to trauma during medical treatment. Fibroblast is a crucial cell for mouth ulcers healing, which undertakes essential functions like synthesis and replenishment of the connective fibers and the amorphous substance during tissue repair (Lendahl et al. 2022). By the primary defence mechanism, the ulcer disappears through the healing process, which is divided into three phases, namely inflammation, proliferation, and remodelling (Toma et al. 2021). In inflammation, neutrophils and macrophages migrate to the ulcer area, resolve respiratory bursts using high oxygen during phagocytosis, and increase reactive oxygen species (ROS), such as superoxide and hydrogen peroxide (Arief and Widodo 2018). Furthermore, high ROS production can cause a pro-oxidant increase and oxidative stress (Bhattacharyya et al. 2014; Phaniendra et al. 2015). This condition interferes the cells communication and causes damage to influence the ulcer healing process (Pisoschi and Pop 2015), such as lengthening the inflammation phase as well as hindering migration process and fibroblast proliferation (Buranasin et al. 2018).

Herbal medicine for the ulcer healing process has been widely used, such as lemongrass (*Cymbopogon citratus* DC) (Veronica et al. 2021). Lemongrass is one of the spices growing in the tropics and is widely used in

Southeast Asia, including Indonesia (Maria et al. 2021). Lemongrass leaf is often discarded without being utilized, whereas its stem serves as a highly valued spice in culinary applications. The leaf contains valuable active compounds, including alkaloids, flavonoids, tannins, steroids, triterpenoids, and saponins, which possess notable antioxidant properties (Comino-Sanz et al. 2021). Natural ingredient with antioxidant content is proven to accelerate the ulcer healing process (Ozougwu 2016) by reducing fibroblast oxidative due to respiratory burst from phagocytic cells (Deng et al. 2021) and to accelerate migration and proliferation (Grgić et al. 2020).

The pharmacological activity of the active compound has bioavailability and absorption limitations in the body that can be controlled with encapsulation technology (Rahim et al. 2022), i.e. by protecting the active compound from oxidation to improve its therapeutic potential (Negi and Kesari 2022). The common polymer material used as a trapping matrix for encapsulation is chitosan (Andikoputri et al. 2021), which is a natural polymer compound obtained from insect exoskeleton (Baharlouei and Rahman 2022), such as horn beetle (*Xylotrupes gideon*) (Veronica et al. 2021). Thus, it can be developed to facilitate a drug delivery system due to its biocompatible, biodegradable, low toxicity level (Agarwal et al. 2018), and simple preparation method (Mohammed et al. 2017). Chitosan physical modification also increases the absorption, diffusion, and penetration to the mucosal layer better than its normal size (Detsi et al. 2020).

Chitosan is a polymer widely used as an active compound trap of a natural ingredient. Previous studies show that *Prunus avium* L. extract encapsulation using nanochitosan and gallic acid can decrease oxidative stress on endothelium cells (Beconcini et al. 2018) and 3T3 fibroblast cells (de Paiva et al. 2021), respectively. Furthermore, the active compound encapsulation of lemongrass with chitosan polymer reduces ROS production of fibroblast by inducing hydrogen peroxide stressor (Fitria et al. 2022). The observation with 2'7' dichlorodihydrofluorescein diacetate (H2DCF-DA) staining using a fluorescent microscope also shows the intensity of green fluorescent cells, indicating reduced ROS production (Andikoputri et al. 2021; Veronica et al. 2021).

This study assesses the activity of chitosan-encapsulated active compounds of lemongrass leaf ethanol exctract. The chitosan was derived from *X. gideon* through the ionic gelation method. It mitigates fibroblast oxidative stress induced by hydrogen peroxide to promote a decrease in oxidative stress. This reduction in oxidative stress can be demonstrated through enhanced migration and proliferation of fibroblast during the healing process in mouth ulcers. Encapsulation of the lemongrass bioactive compounds is an innovative approach allowing protection against oxidation, thermal degradation and increasing bioavailability. Encapsulation is promising to improve the performance of medicines in oral health, such as mouthwashes.

2. Materials and Methods

2.1. Polymeric materials

The source of chitosan in this study was *X. gideon* obtained from Cangkurawok, Damaga, and Balumbang Jaya, Bogor, East Java. All parts of *X. gideon* body were detached, followed by drying for five days, and then continied to the processes of demineralization (3N HCl), deproteinization (3N NaOH), discoloration (4% H_2O_2) and deacetylation (50% NaOH) (Komariah et al. 2019).

2.2. Lemongrass extract (LE) preparation

Lemongrass (*Cymbopogon citratus*) was collected from Balai Penelitian Tanaman Rempah dan Obat (BALITRO), Indonesian Medicinal and Aromatic Crops Research Institute (IMACRI), West Java, Indonesia. The determination was carried out at Pusat Riset Biologi, Badan Riset dan Organisasi Nasional (BRIN), Cibinong, West Java, Indonesia. The leaves were dried in an oven at a temperature of 45 °C for one week and extracted using the maceration method (Felicia et al. 2022). They were soaked in 70% ethanol at a 1:10 (w/v) ratio for 24 h at room temperature. The macerated mixture was filtered using a filter paper and evaporated with a rotary vacuum evaporator at a temperature of 40 °C at 100 rpm for 2 h (Fitria et al. 2022).

2.3. The preparation of LE-loaded on chitosan X. gideon (LEChXg)

Chitosan with 83% degrees of deacetylation and a weight value of 0.5 g was dissolved in 1% acetic acid (Merck, Germany). Subsequently, 2 mL of 10% lemongrass leaf extract (LE) and 100 mL of distilled water were added (Veronica et al. 2021). Stirring was conducted using a magnetic stirrer (IKA RH basic 2, Germany) and heating at 40 °C with a speed of 2,500 rpm for 20 min and was subsequently carried out without heating for 100 min. Following the previous step, 40 mL of 0.1% tripolyphosphate (Sigma-Aldrich, USA) was added dropwise while stirring for one hour. Subsequently, 0.1 mL of 0.1% Tween 80 (Merck, France) was introduced, and the mixture was stirred again at a speed of 2,500 rpm for 30 min (Andikoputri et al. 2021; Veronica et al. 2021). The particle size of lemongrass extract was determined using a particle size analyzer (PSA) (Horiba Scientific, Nano Particle Analyzer SZ-100, UK) (Budi et al. 2020).

2.4. Culture of fibroblast

Human dermal fibroblast (HDF) was obtained from the Biorepository of Stem Cell Research Center, Yarsi University, Indonesia (Fitria et al. 2022). Fibroblast was planted in a cell culture dish and incubated at 37 °C with 5% CO_2 for 24 h. The growth medium was replaced by media containing DMEM, 10% FBS, and antibiotic-antimycotic (penicillin, streptomycin, and amphotericin B). The cultured cells were divided into eight treatment groups as follows: (1) without any treatment or stressor, (2) treated with hydrogen peroxide stressor (H_2O_2) as a negative con-

trol, (3) treated with H_2O_2 plus ascorbic acid as a positive control, (4–8) treated with H_2O_2 plus nanochitosan-encapsulated lemongrass (LEChXg) at concentrations of 100, 200, 300, 400, and 500 µg/mL, respectively. The stressor was given as the addition of 100 µM H_2O_2 (Lionetti et al. 2019) followed by incubation at 37 °C with 5% CO_2 for 60 min (Fitria et al. 2022).

2.5. Proliferation assay

Fibroblasts planted at a density of 1 × 10^3 cells/well in a 96-well plate were incubated at 37 ºC for 24 h. After 24, 48, and 72 h of treatment with a various concentration of LEChXg, the cells were washed by PBS 1×, and 100 µL of CCK-8 solution was added. The absorbance was measured at 450 nm using a microplate reader (Tecan Group Ltd. Mannedorf, Switzerland) and the percentage of fibroblast proliferation was calculated as shown below (Felicia et al. 2022).

$$proliferation\,rate\,(\%) = \frac{absorbance\ of\ sample}{absorbance\ of\ negative\ control} \times 100 \quad (1)$$

2.6. Migration assay

The cells were planted in a 24-well plate (2.9 × 10^3 cells/well) (Kauanova et al. 2021) and incubated until confluent. After reaching confluency, the monolayer was scratched gently using a white tip perpendicularly to the bottom of the monolayer. After the first scratch was conducted, a second scratch was made by crossing the first one. Thus, a cross pattern would be formed. Next, the cells were washed in PBS once and subsequently were treated (Felicia et al. 2022). The cell migration was observed after 0, 2, 4, 6, 24, and 48 h after the that and photographed using a microscope (EVOS FLc Cell Imaging System). At the end of the experiment, the wound closure was analyzed using ImageJ.

2.7. Data Analysis

Statistical quantification was conducted using SPSS version 2.3 and the data were presented as mean ± standard deviation (SD). Meanwhile, MANOVA was used to compare the groups against several dependent variables (times) in migration and proliferation assays. For a significant difference ($p < 0.05$), Post Hoc's Tukey test was performed.

3. Results and Discussion

3.1. Characteristics of LEChXg particle

Characteristic of LEChXg particle was used to estimate and determine the particle size and distribution of particle size. Meanwhile, particle size was measured using a PSA with a repetition of three times. The result of particle measurement and polydispersity index (PDI) LEChXg is shown in Table 1.

TABLE 1 Characterization of LEChXg nanoparticles.

Sample	Particle size (nm)	PDI	Zeta Potential (mV)
LEChXg	489.57 ± 3.44	0.69 ± 0.06	31.2 ± 0.87

PDI: poly dispersity index.

The LEChXg particle measurement indicated that the average size was 489.57 nm. Therefore, LEChXg fulfilled the requirements as a nanoparticle with a size range between 50–500 nm particle (Ismail and Harun 2019). According to Idacahyati et al. (2021), a nano-size particle should range from 1–1000 nm. PDI is a value that shows particle size distribution with a range of 0–1. A sample with a bigger and smaller size range has higher PDI values (Karmakar 2019). The result of particle distribution with PDI values 0.035 to 0.05 is considered to have monodisperse particle distribution (Clayton et al. 2016). Polydispersity is a macromolecule with various good weights, sizes, and mass distribution (Kim et al. 2019). The zeta potential value of LEChXg was 31.2 mV. Nanoparticles with zeta potential values smaller than 31.2 mV and greater than +30 mV also indicated good stability (Prakash et al. 2014). A dispersion system with a small zeta potential value was easier to form, such as the Van der Waals style in particle interaction (Juliantoni et al. 2020).

3.2. Migration of fibroblast

The migration test was conducted using scratch by making an artificial gap (scratch) in confluent monolayer cells. The gap allowed cells to communicate with each other. It also showed the ability of fibroblasts to move toward the ulcer, carry out proliferation, and form an extracellular matrix. The results of fibroblast migration showed that migration in 0 and 2 h did not indicate a significant difference ($p = 0.222$). However, at the observations of 4 and 6 h, there was a significant difference ($p < 0.05$) between the group with a stressor and the LEChXg group at a concentration of 300 µg/mL. The group treated with LEChXg showed a good migration activity by closing the most significant gap compared to those treated with a H_2O_2 stressor only. At 24 h, all study groups had no significant difference ($p > 0.05$). However, the fibroblast group treated with stressor showed less optimal migration due to an open gap of 0.51 ± 0.88 µm^2. The average fibroblast migration ability is shown in Table 2. Microscopic observation of the cell migration can be seen in Figure 1.

The observation of fibroblast migration when closing the gap is related to the ulcer healing process. The inflammation cells are stimulated to release various mediators (Chen et al. 2018) and growth factors such as the transforming growth factor-beta 1 (TGF-β) factor of fibroblast when oxidative stress is reduced (Jimi et al. 2020). H_2O_2 is one of the most critical compounds in ROS signaling studies due to its physicochemical properties, relatively low reactivity, and ability to diffuse through membranes. The addition at low concentrations increases intracellular ROS levels without causing oxidative stress. It also increases the migration of mesenchymal cells through extracellular signal-regulated kinases (ERK) 1/2 and focal adhesion kinase (FAK) pathways. Cellular abnormalities are increased when cells experience oxidative stress (Waheed

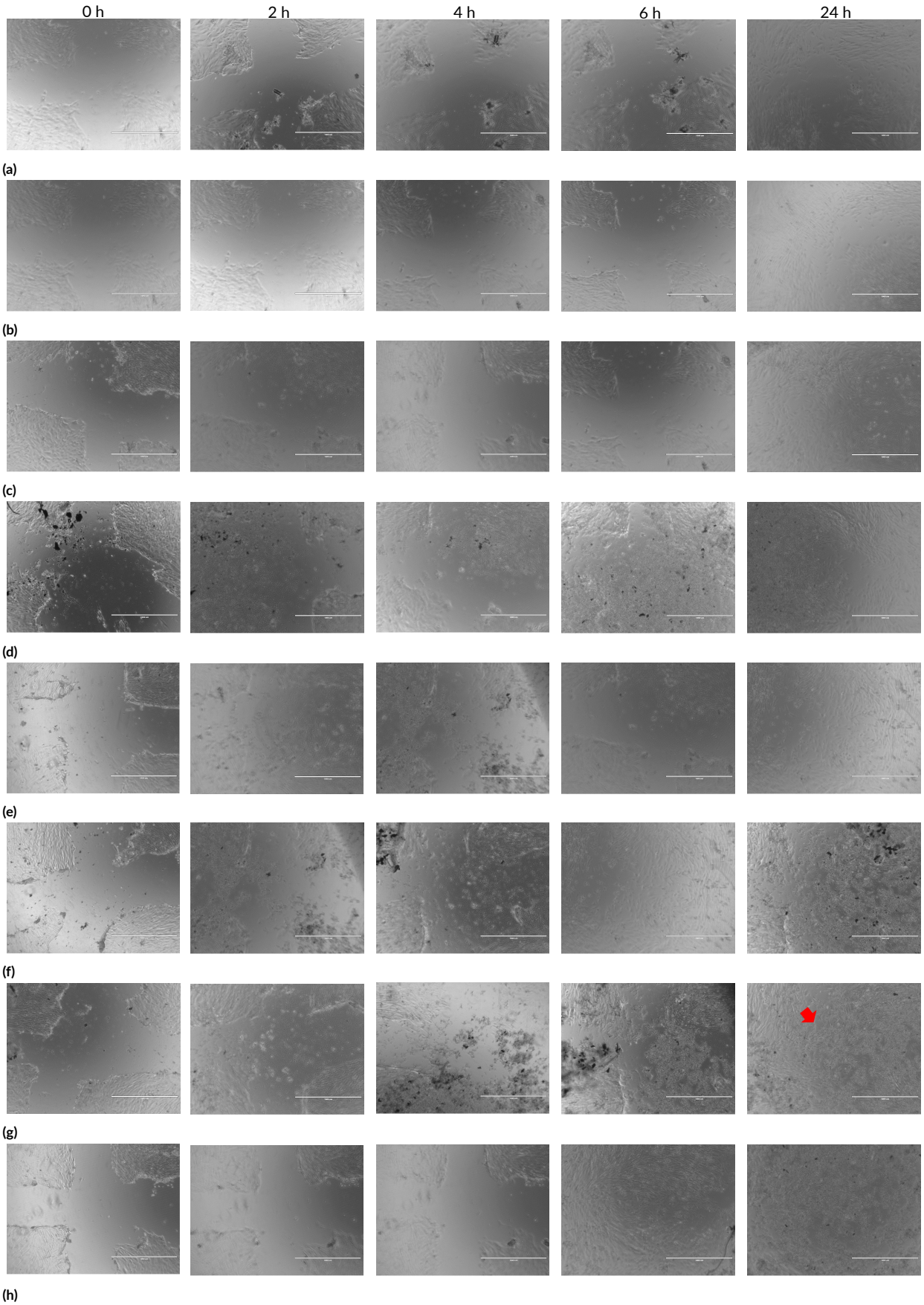

FIGURE 1 Fibroblast migration at 0, 2, 4, 6, and 24-h observation after the scratch wound. The yellow arrow shows a shaped artificial gap. The red arrow shows the artificial gap closing caused by the fibroblast migration. A: negative control, B: H_2O_2 only. C-H received H_2O_2 with treatment as follows: C: ascorbic acid as positive control, D: LEChXg 100 µg/mL, E: LEChXg 200 µg/mL, F. LEChXg 300 µg/mL, G. LEChXg 400 µg/mL, and H LEChXg 500 µg/mL. The observation was at 1,000× magnification. Scale bar = 1000µm.

TABLE 2 Fibroblast migration.

Group	Measurement number (n)	Migration (μm^2)				
		0 h	2 h	4 h	6 h	24 h
Untreated	3	2.87 ± 0.14	2.71 ± 0.28	2.53 ± 0.18^{ab}	2.50 ± 0.18^{ab}	0.00 ± 0.00
H_2O_2	3	3.82 ± 0.88	3.75 ± 0.91	3.41 ± 1.04^{a}	3.21 ± 1.04^{a}	0.51 ± 0.88
H_2O_2 + Ascorbic Acid	3	2.99 ± 0.20	2.82 ± 0.18	2.74 ± 0.19^{ab}	2.61 ± 0.17^{ab}	0.00 ± 0.00
H_2O_2 + LEChXg 100 µg/mL	3	2.70 ± 0.27	2.55 ± 0.28	2.24 ± 0.49^{ab}	1.88 ± 0.33^{ab}	0.00 ± 0.00
H_2O_2 + LEChXg 200 µg/mL	3	3.31 ± 0.05	2.89 ± 0.23	2.58 ± 0.18^{ab}	2.49 ± 0.18^{ab}	0.00 ± 0.00
H_2O_2 + LEChXg 300 µg/mL	3	2.92 ± 0.08	2.61 ± 0.18	1.44 ± 0.20^{b}	1.38 ± 0.16^{a}	0.00 ± 0.00
H_2O_2 + LEChXg 400 µg/mL	3	3.99 ± 1.73	3.43 ± 1.50	3.16 ± 1.38^{ab}	2.55 ± 1.11^{ab}	0.00 ± 0.00
H_2O_2 + LEChXg 500 µg/mL	3	2.68 ± 0.04	2.32 ± 0.16	2.03 ± 0.21^{ab}	1.94 ± 0.21^{ab}	0.00 ± 0.00

a-b in different columns migration shows a significant difference ($p < 0.05$), The superscript 'a' indicates a higher migration compared to group 'b,' while 'ab' indicates migration that is not different from groups 'a' and 'b'.

et al. 2022). Buranasin et al. (2018) stated that gingival fibroblast exposed to high glucose concentrations causes oxidative stress by increasing ROS production and inhibiting the migration process associated with inhibiting basic fibroblast growth factor (bFGF) signaling.

3.3. Fibroblast proliferation

The results of fibroblast proliferation in 24 h showed that the group treated with stressor was significantly different ($p < 0.05$) from those without stressor, as well group treated with ascorbic acid as a non-enzymatic antioxidant and LEChXg. The group treated with stressors indicated low cell proliferation compared to the others. Hydrogen peroxide is a molecule with low reactivity but can easily penetrate the cell membrane, generating the most reactive type of oxygen, hydroxyl radical, and converting Fe^{2+} atau Cu^{+} to OH (Nita and Grzybowski 2016).

Fibroblast proliferation without stressors had the highest proliferative compared to the other groups. The average proliferation is shown in Figure 2. Fibroblast can counteract an elevation in free radicals by augmenting the synthesis of endogenous antioxidants, thereby preventing any adverse impact on the proliferation of stressor-unexposed cells (Tsuneda 2020) The group treated with ascorbic acid showed good proliferation after exposing the cells to stressors H_2O_2. Ascorbic acid is an antioxidant that can neutralize oxidative stress by donating an electron to prevent other oxidized compounds and scavenging superoxide anion, hydroxyl radical, and lipid hydroperoxide (Pehlivan 2017).

Fibroblast proliferation in 48 h of observation showed that the group treated with H_2O_2 is significantly different ($p < 0.05$) from those without stressor, as well as the group treated with ascorbic acid, and LEChXg concentrations at 200, 300, and 400 µg/mL. Meanwhile, the group with stressors did not report a significant difference ($p > 0.05$) from LEChXg 100 and 500 µg/mL.

The group treated with H_2O_2 at 48 h showed a decrease in proliferation compared to the 24-h observation. It indicates that the cells experienced oxidative stress could not detoxify or repair the damage resulting from free radicals (Phaniendra et al. 2015). Therefore, it caused cell damage and affected proliferation. The ascorbic acid group and those treated with LEChXg at all the the tested concentra-

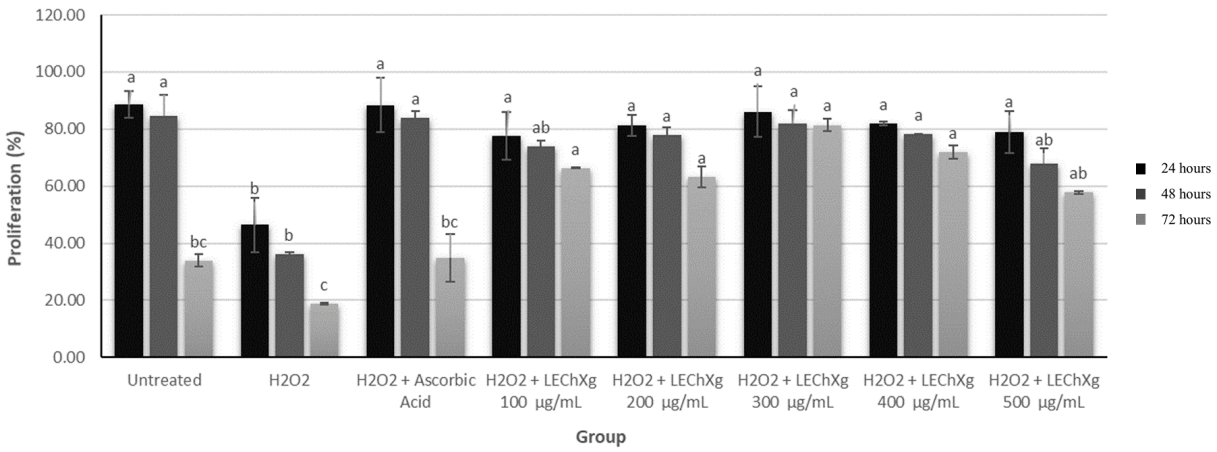

FIGURE 2 Fibroblast proliferation. a-c in different hours shows a significant difference ($p < 0.05$) The superscript 'a' indicates higher proliferation compared to groups 'b' and 'c,' while 'b' indicates higher proliferation than group 'c.' Superscript 'ab' implies no significant difference between groups 'a' and 'b,' and superscript 'bc' no significant difference between groups 'b' and 'c'.

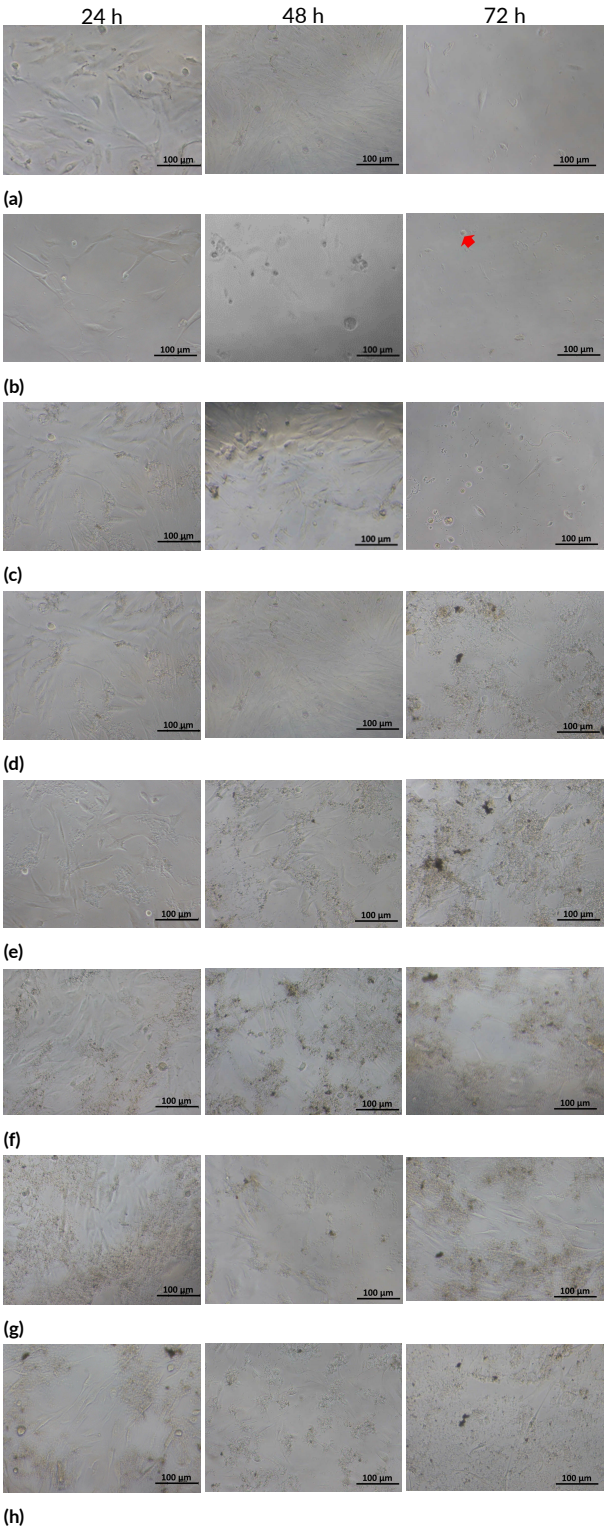

FIGURE 3 Fibroblast proliferation at 24, 48, and 72-h observation as indicated. The yellow arrow shows the fibroblast, which is living with a clearly visible cell nucleus, while the red arrow shows a dead fibroblast by shrinking in the cells. A: negative control, B: H_2O_2 only. C-H received H_2O_2 with treatment as follows: C: ascorbic acid as positive control, D: LEChXg 100 µg/mL, E: LEChXg 200 µg/mL, F. LEChXg 300 µg/mL, G. LEChXg 400 µg/mL, and H LEChXg 500 µg/mL. The observation was at 1,000× magnification.

tions could detoxify or repair the damages resulting from increased free radicals to enable a relatively stable proliferation.

The observation at 72 h showed that the group treated with stressor was significantly different ($p < 0.05$) from LEChXg concentrations 100, 200, 300, and 400 µg/mL. The difference showed increased proliferation higher than the group treated with a stressor. In contrast, the non-stressor, ascorbic acid, and LEChXg 500 µg/mL groups showed no significant difference ($p = 1.000$). Even though there was no difference, these groups exhibited higher proliferation cells than those treated with stressors. The proliferation of fibroblast in 24, 48, and 72 h observation is shown in Figure 3.

The results of the proliferation of the non-stressor, stressor, and ascorbic acid groups experienced decreased proliferation. However, a proliferation of the group treated with LEChXg was relatively stable. LEChXg maintained or stabilized proliferation until 72 h observation of fibroblast, which experienced oxidative stress. Pan et al. (2022) showed that active compounds of lemongrass increase cell proliferation power by reducing oxidative stress resulting from high ROS. Similarly, Roriz et al. (2014) indicated that lemongrass showed an antioxidant effect by improving the superoxide dismutase enzyme (SOD) activity and reducing the production of ROS in macrophages. Chitosan plays a role in scavenging free radicals and inhibiting oxidative damage (Pellis et al. 2022). The leading functional group, such as hydroxyl and an amino groups can reduce free radicals after a reaction at C-2, C-3, and C-6 positions of the pyranose ring to produce a stable macromolecule (Muthu et al. 2021). At the end of our observation, there was an aggregate formed in the LEChXg group due to polydisperse particle distribution with various molecular weights, sizes, and mass distributions, as well as zeta potential value which was relatively greater than +30 mV (Dipahayu and Kusumo 2021).

Our results were consistent with Beconcini et al. (2018), where the encapsulation of *Prunus avium* extract with chitosan and its derivatives reduces oxidative stress in human umbilical vein endothelial cells (HUVEC). An in vivo study on rat liver cells showed that encapsulated *Pinus merkusii* extract with chitosan reduces malondialdehyde (MDA) levels. An increase in MDA levels indicates hepatocyte cell membrane damage after exposure to ROS. Meanwhile, the decrease showed that the encapsulation of *P. merkusii* extract inhibits ROS production (Di Santo et al. 2021).

Damage in the epithelium and lamina propria as connective tissue with a predominance of fibroblast in mouth ulcers accelerated the healing process by encapsulating lemongrass leaf extract ethanol with chitosan *X. gideon*. The acceleration of the process was determined through increased migration and proliferation of fibroblasts after experiencing oxidative stress during the inflammatory process.

4. Conclusions

In conclusion, encapsulation of lemongrass leaf extract ethanol with chitosan *X. gideon* reduced fibroblast oxidative stress and was shown with good migration and proliferation at a range of 100–500 µg/mL, with the best concentrations at 300 µg/mL.

Acknowledgments

The authors are grateful to the Universitas Trisakti study institute for supporting the fund. The authors are also grateful to the Terpadu Laboratory in the Faculty of Medicine, Universitas Yarsi, BIOCORE and MICORE Laboratory in the Faculty of Dentistry, Universitas Trisakti, for the facilities provided.

Authors' contributions

KK designed the study, analysed the data, carried out the laboratory work and wrote the manuscript; PT wrote the manuscript; RW wrote the manuscript and analysed the data; NE carried out the laboratory work; DN wrote the manuscript. All authors read and approved the final version of the manuscript.

Competing interests

The authors declare no conflict of interest concerning the present paper.

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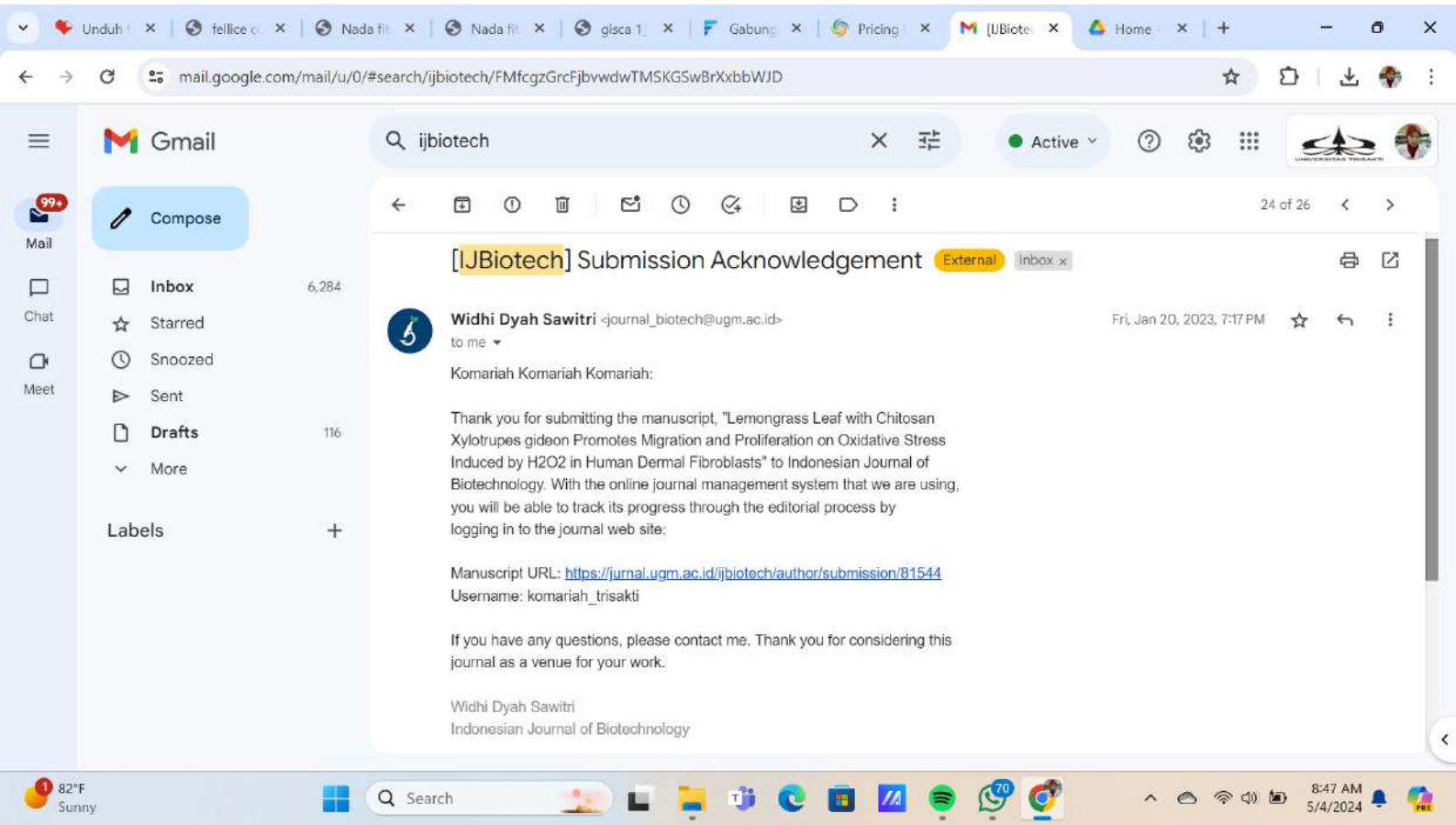
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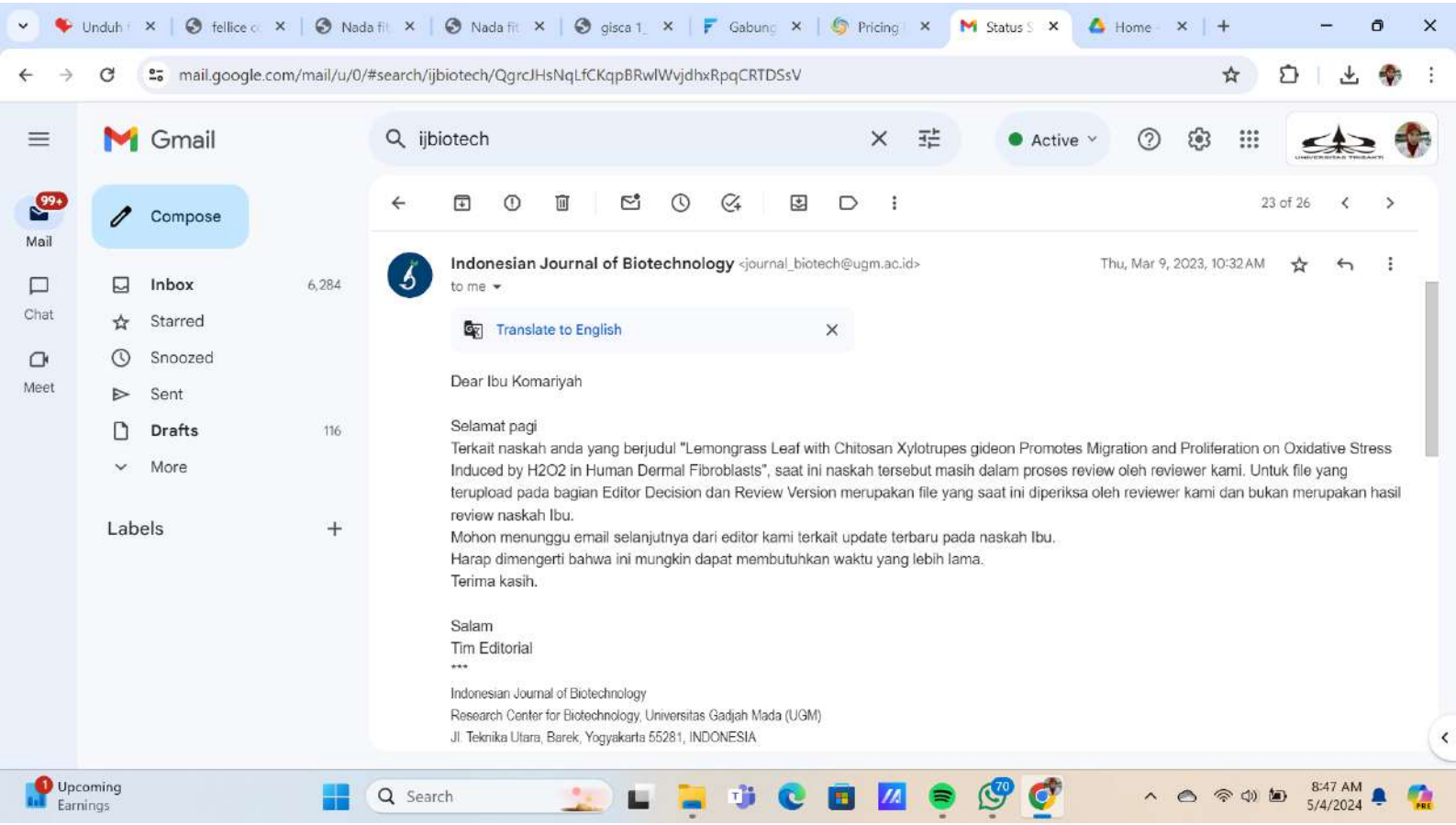

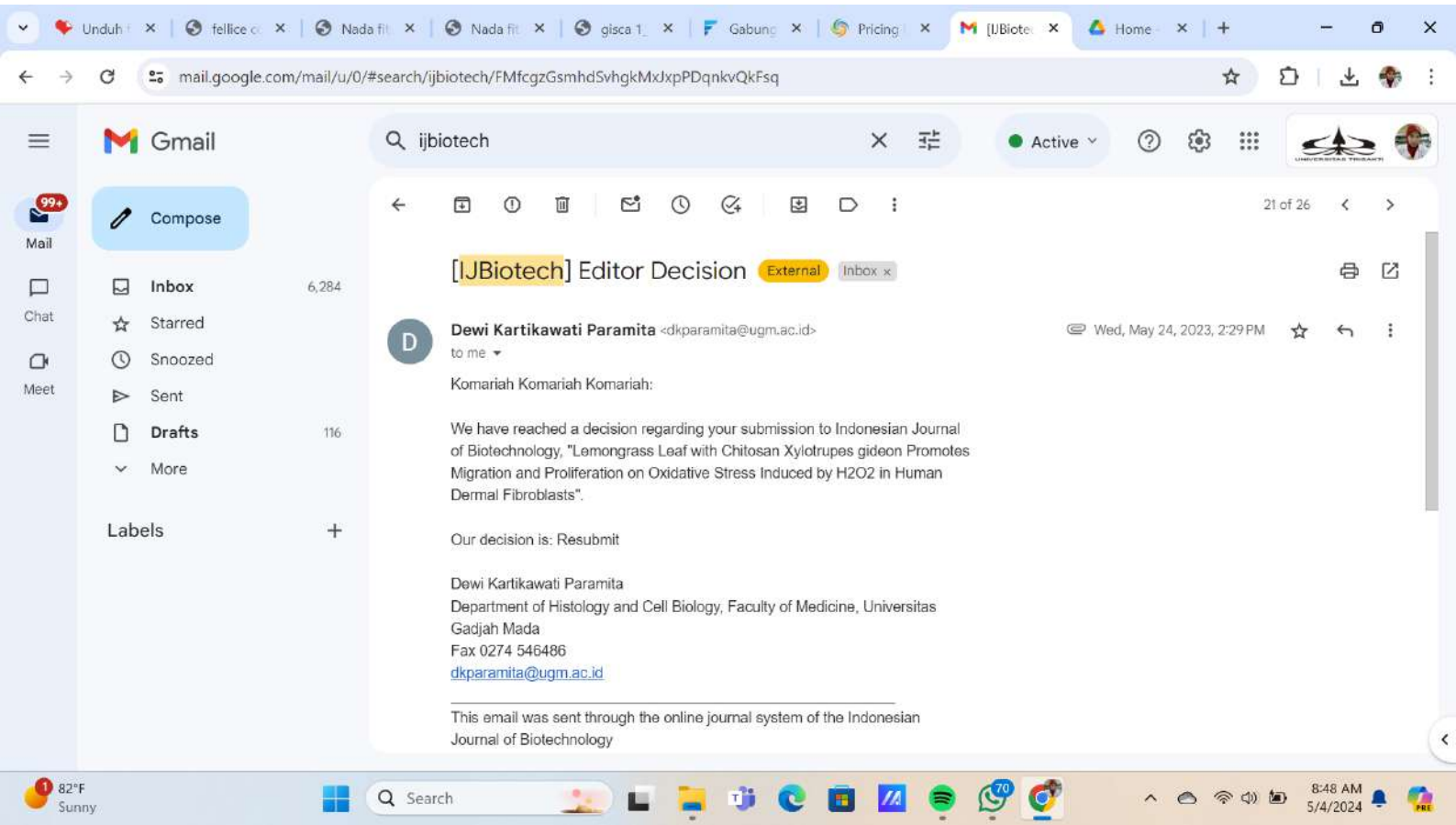
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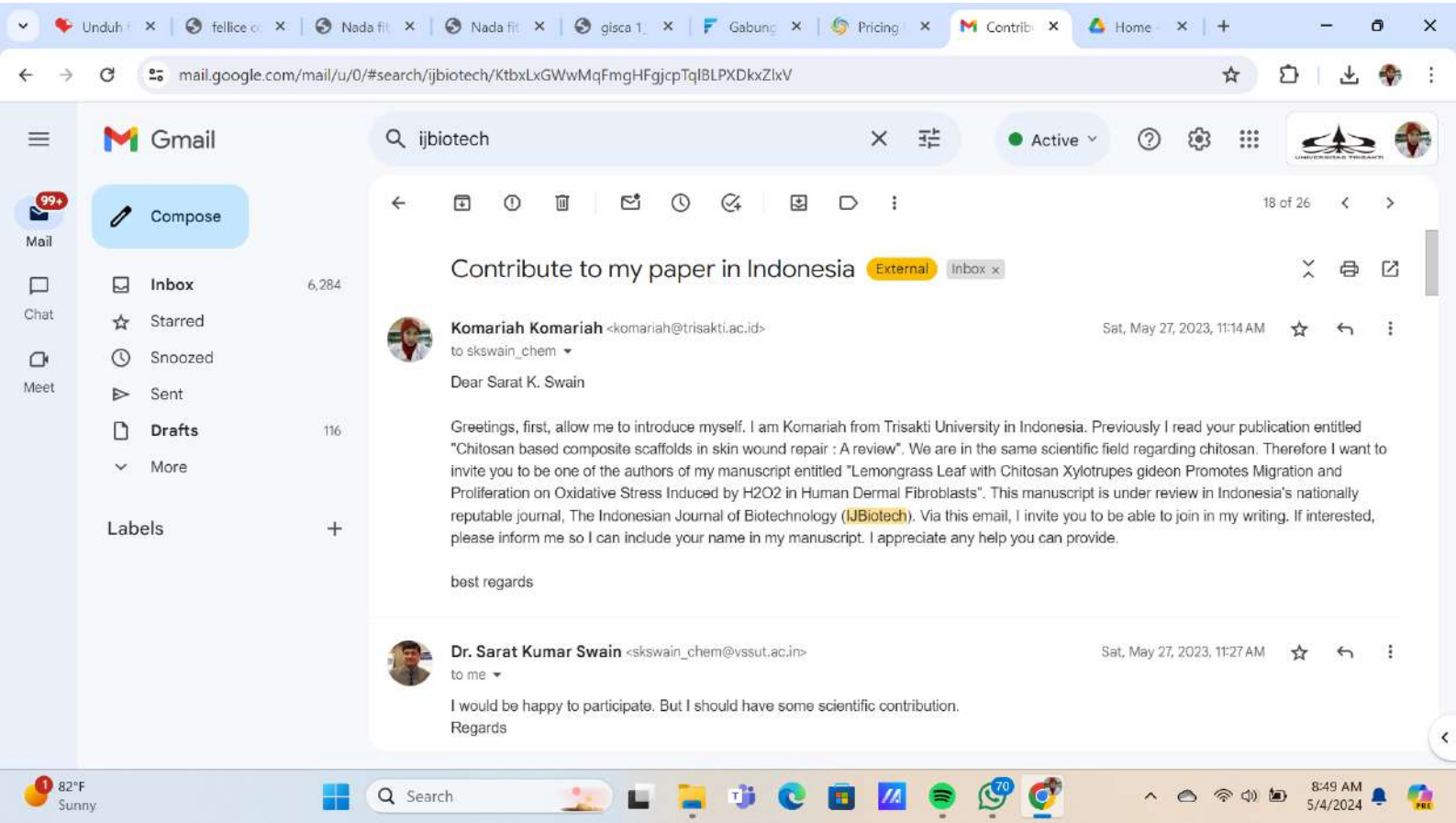
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Greetings, first, allow me to introduce myself. I am Komariah from Trisakti University in Indonesia. Previously I read your publication entitled "Chitosan based composite scaffolds in skin wound repair : A review". We are in the same scientific field regarding chitosan. Therefore I want to invite you to be one of the authors of my manuscript entitled "Lemongrass Leaf with Chitosan Xylotrupes gideon Promotes Migration and Proliferation on Oxidative Stress Induced by H2O2 in Human Dermal Fibroblasts". This manuscript is under review in Indonesia's nationally reputable journal, The Indonesian Journal of Biotechnology (IJBiotech). Via this email, I invite you to be able to join in my writing. If interested, please inform me so I can include your name in my manuscript. I appreciate any help you can provide.
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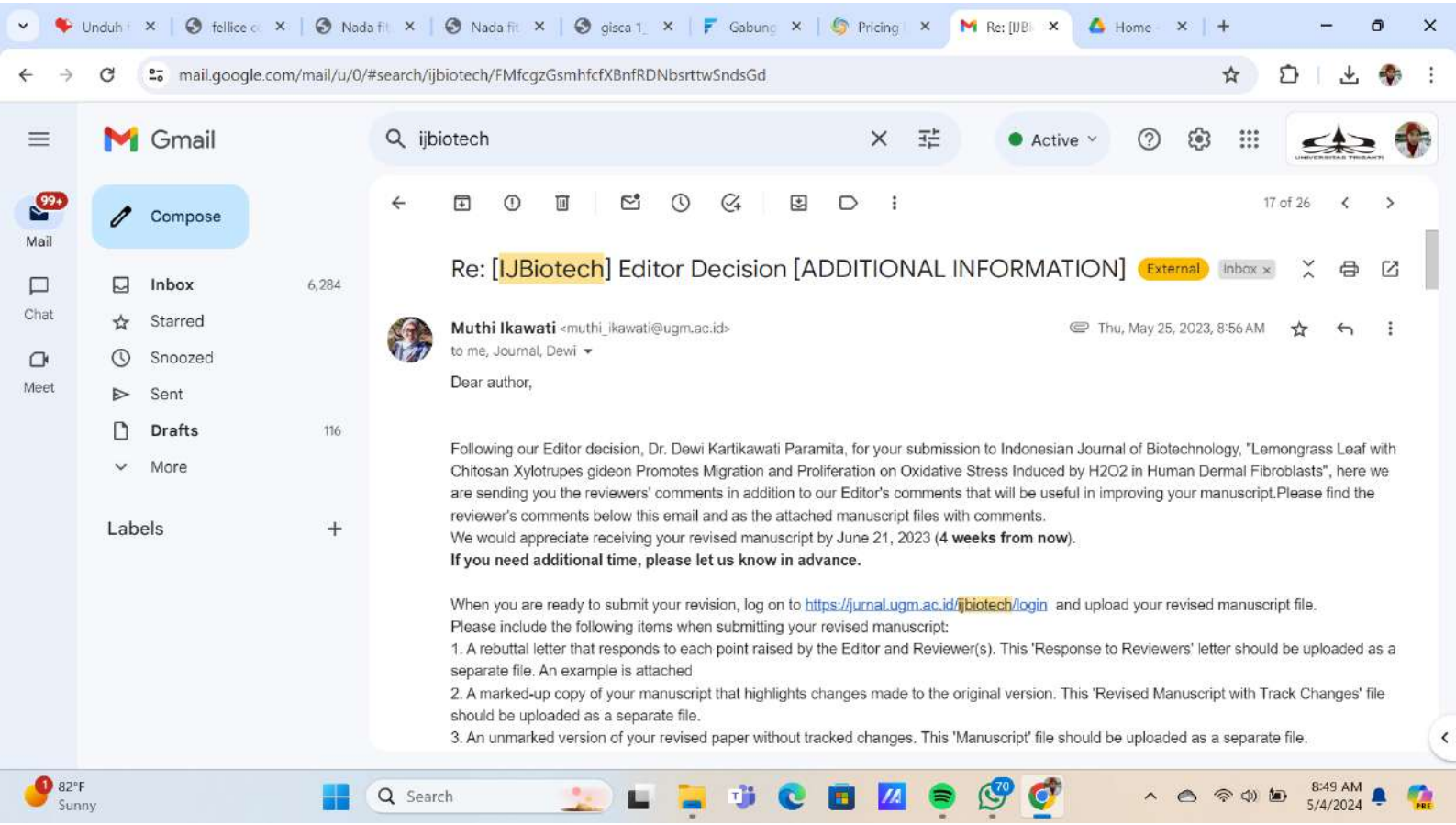
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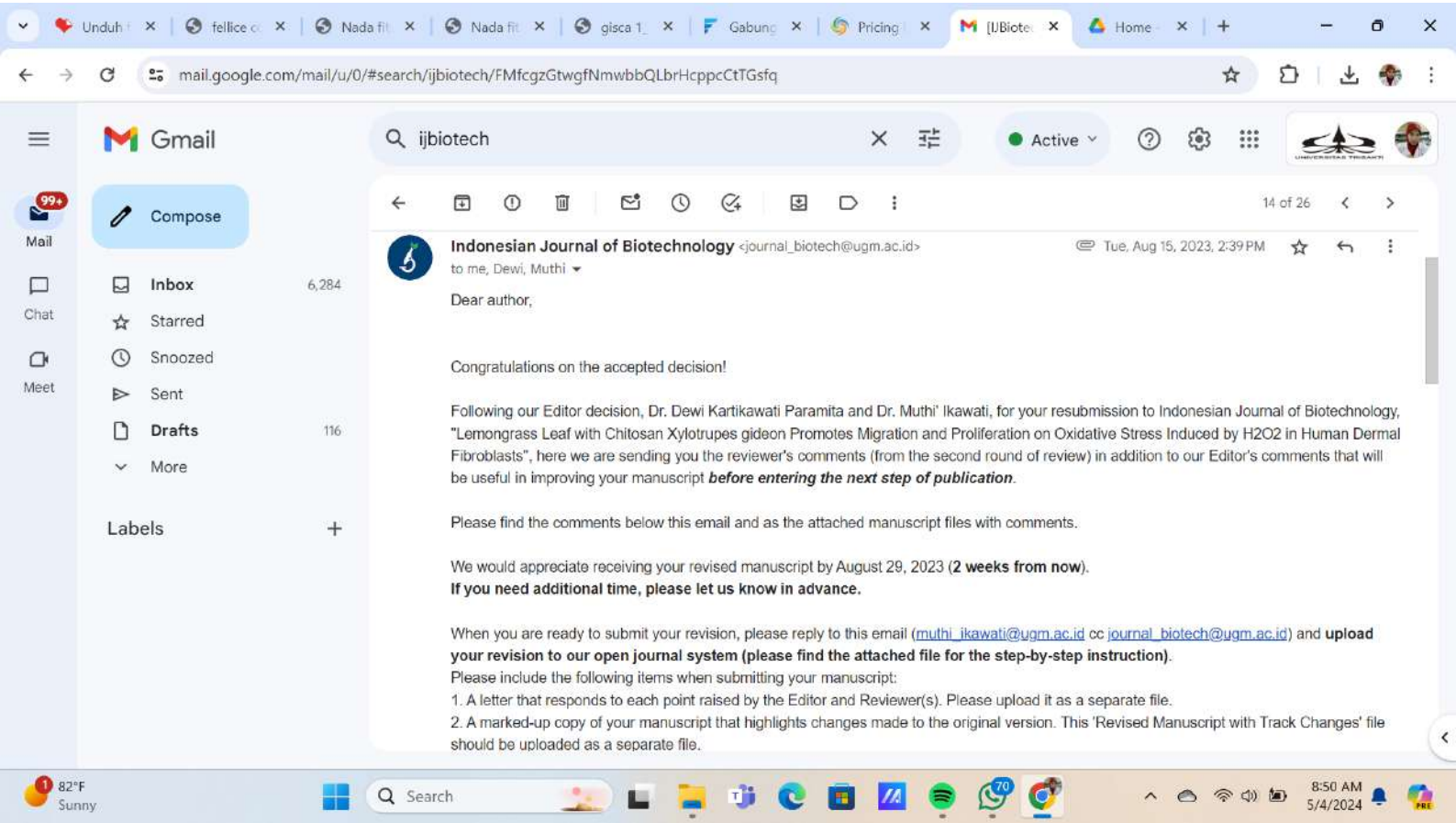
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Dear author,
Congratulations on the accepted decision!
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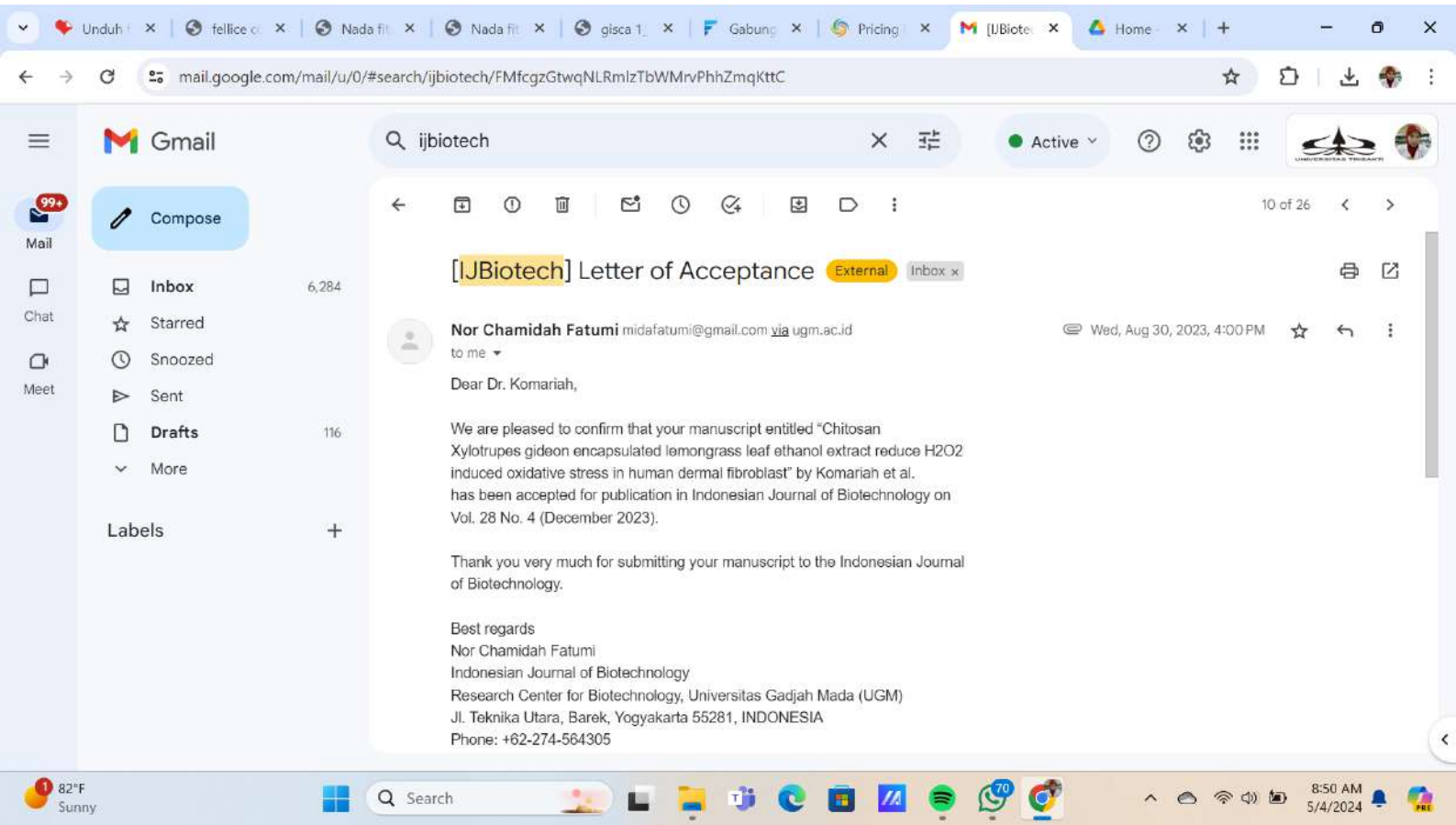
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Dear Dr. Komariah,
We are pleased to confirm that your manuscript entitled "Chitosan Xylotrupes gideon encapsulated lemongrass leaf ethanol extract reduce H2O2 induced oxidative stress in human dermal fibroblast" by Komariah et al. has been accepted for publication in Indonesian Journal of Biotechnology on Vol. 28 No. 4 (December 2023).
Thank you very much for submitting your manuscript to the Indonesian Journal of Biotechnology.
Best regards
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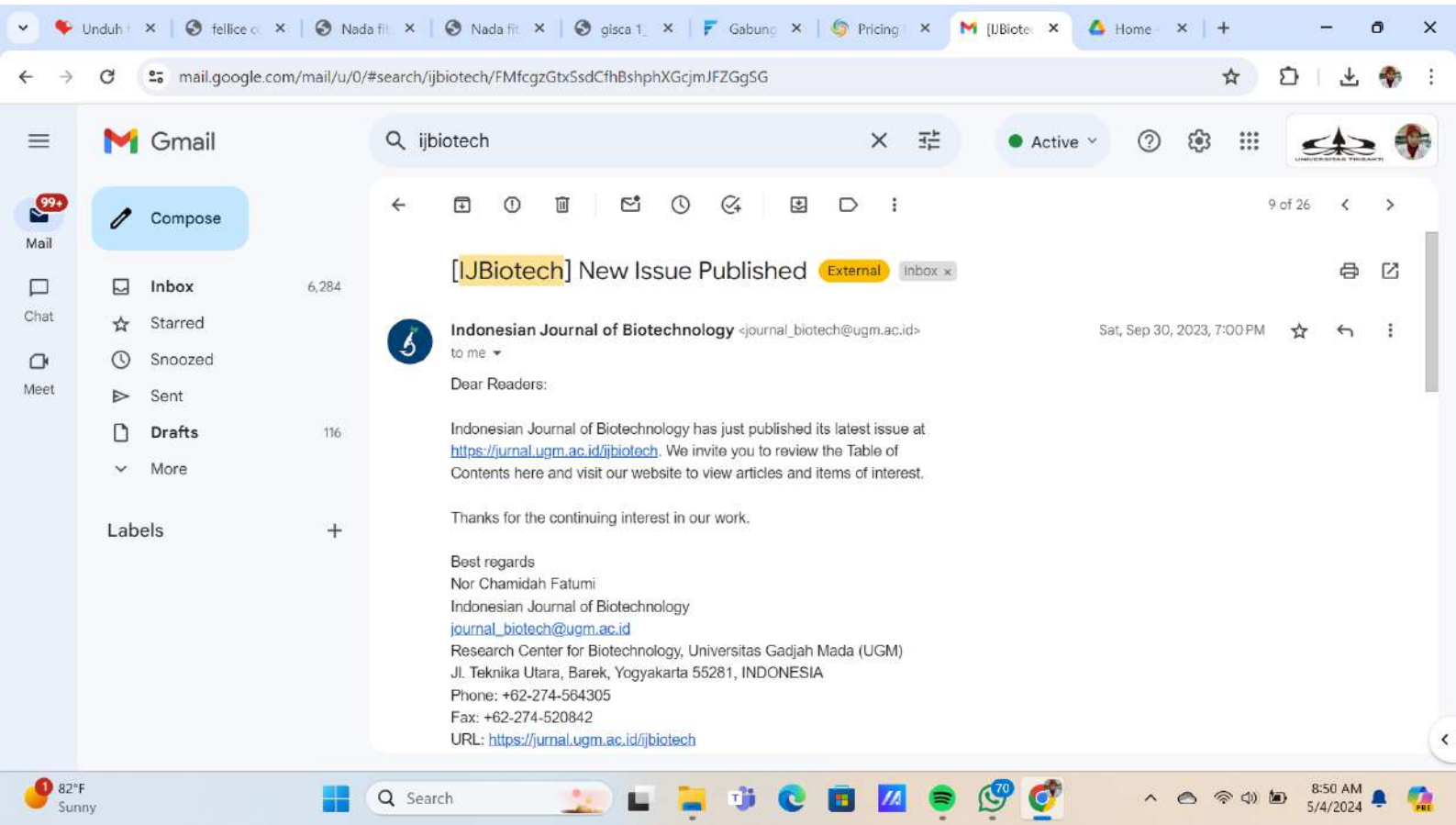
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VOLUME 28(4), 2023, 191-199 | RESEARCH ARTICLE

Chitosan *Xylotrupes gideon* encapsulated lemongrass leaf ethanol extract reduce H_2O_2-induced oxidative stress in human dermal fibroblast

Komariah Komariah[1,*], Pretty Trisfilha[2], Rahman Wahyudi[2], Nada Erica[3], Didi Nugroho[4], Yessy Ariesanti[5], Sarat Kumar Swain[6]

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[2] Department of Oral Biology sub-division of Oral Pathology, Faculty of Dentistry, Universitas Trisakti, Jakarta 11440, Indonesia
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SUBMITTED 20 January 2023 **REVISED** 4 July 2023 **ACCEPTED** 15 August 2023

ABSTRACT During phagocytosis, phagocyte cells discharge reactive oxygen species referred to as respiratory bursts, inducing a rise in pro-oxidants and subjecting the cell to oxidative stress. Such stress is a biological mechanism related to an imbalance in pro-oxidant/antioxidant homeostasis, which generates toxic reactive oxygen. Encapsulation is a coating process to improve the stability of bioactive compounds from lemongrass extract. Therefore, this study aims to determine the encapsulation activity of lemongrass leaf extract with chitosan *X. gideon* (LEChXg) to reduce the oxidative stress of fibroblasts. The research used the human dermal fibroblast (HDF) cell line, comprising negative and positive controls and use of LEChXg 100, 200, 300, 400, and 500 µg/mL. HDF cell migration was evaluated by employing the scratch wound healing method and the wound closure was oberseved at 0, 2, 4, 6, and 24 h intervals. The cell proliferation was observed at 24, 48, and 72 h using CCK-8 at a 450 nm wavelength. The results showed that the observations at 0, 2, and 4 h did not demonstrate any significant difference on the cell migration ($p > 0.05$) among the groups. However, the wound closure at 4 and 6 h showed a significant difference ($p < 0.05$) with LEChXg 300 µg/mL. Despite the lack of any significant variation observed up to 24 h, fibroblast subjected to the stressor did not achieve complete closure. The groups treated with LEChXg were more stable in maintaining fibroblast proliferation up to the end of the observation than those with stressors at 24, 48, and 72 h. Fibroblast induced with a stressor was also more stable in maintaining migration and proliferation in groups receiving LEChXg 300 µg/mL.

KEYWORDS Chitosan *X. gideon*; Lemongrass leaf; Migration; Oxidative stress; Proliferation

1. Introduction

Mouth or oral ulcers are generally known as discontinuities of oral mucosa characterized by epithelial tissue damage and connective tissue in lamina propria of the mucosa in oral cavity (Zakiawati et al. 2020). About 40% of people have been estimated to suffer from oral ulcers disease (Zakiawati et al. 2020) due to trauma during medical treatment. Fibroblast is a crucial cell for mouth ulcers healing, which undertakes essential functions like synthesis and replenishment of the connective fibers and the amorphous substance during tissue repair (Lendahl et al. 2022). By the primary defence mechanism, the ulcer disappears through the healing process, which is divided into three phases, namely inflammation, proliferation, and remodelling (Toma et al. 2021). In inflammation, neutrophils and macrophages migrate to the ulcer area, resolve respiratory bursts using high oxygen during phagocytosis, and increase reactive oxygen species (ROS), such as superoxide and hydrogen peroxide (Arief and Widodo 2018). Furthermore, high ROS production can cause a pro-oxidant increase and oxidative stress (Bhattacharyya et al. 2014; Phaniendra et al. 2015). This condition interferes the cells communication and causes damage to influence the ulcer healing process (Pisoschi and Pop 2015), such as lengthening the inflammation phase as well as hindering migration process and fibroblast proliferation (Buranasin et al. 2018).

Herbal medicine for the ulcer healing process has been widely used, such as lemongrass (*Cymbopogon citratus* DC) (Veronica et al. 2021). Lemongrass is one of the spices growing in the tropics and is widely used in

Southeast Asia, including Indonesia (Maria et al. 2021). Lemongrass leaf is often discarded without being utilized, whereas its stem serves as a highly valued spice in culinary applications. The leaf contains valuable active compounds, including alkaloids, flavonoids, tannins, steroids, triterpenoids, and saponins, which possess notable antioxidant properties (Comino-Sanz et al. 2021). Natural ingredient with antioxidant content is proven to accelerate the ulcer healing process (Ozougwu 2016) by reducing fibroblast oxidative due to respiratory burst from phagocytic cells (Deng et al. 2021) and to accelerate migration and proliferation (Grgić et al. 2020).

The pharmacological activity of the active compound has bioavailability and absorption limitations in the body that can be controlled with encapsulation technology (Rahim et al. 2022), i.e. by protecting the active compound from oxidation to improve its therapeutic potential (Negi and Kesari 2022). The common polymer material used as a trapping matrix for encapsulation is chitosan (Andikoputri et al. 2021), which is a natural polymer compound obtained from insect exoskeleton (Baharlouei and Rahman 2022), such as horn beetle (*Xylotrupes gideon*) (Veronica et al. 2021). Thus, it can be developed to facilitate a drug delivery system due to its biocompatible, biodegradable, low toxicity level (Agarwal et al. 2018), and simple preparation method (Mohammed et al. 2017). Chitosan physical modification also increases the absorption, diffusion, and penetration to the mucosal layer better than its normal size (Detsi et al. 2020).

Chitosan is a polymer widely used as an active compound trap of a natural ingredient. Previous studies show that *Prunus avium* L. extract encapsulation using nanochitosan and gallic acid can decrease oxidative stress on endothelium cells (Beconcini et al. 2018) and 3T3 fibroblast cells (de Paiva et al. 2021), respectively. Furthermore, the active compound encapsulation of lemongrass with chitosan polymer reduces ROS production of fibroblast by inducing hydrogen peroxide stressor (Fitria et al. 2022). The observation with 2'7' dichlorodihydrofluorescein diacetate (H2DCF-DA) staining using a fluorescent microscope also shows the intensity of green fluorescent cells, indicating reduced ROS production (Andikoputri et al. 2021; Veronica et al. 2021).

This study assesses the activity of chitosan-encapsulated active compounds of lemongrass leaf ethanol exctract. The chitosan was derived from *X. gideon* through the ionic gelation method. It mitigates fibroblast oxidative stress induced by hydrogen peroxide to promote a decrease in oxidative stress. This reduction in oxidative stress can be demonstrated through enhanced migration and proliferation of fibroblast during the healing process in mouth ulcers. Encapsulation of the lemongrass bioactive compounds is an innovative approach allowing protection against oxidation, thermal degradation and increasing bioavailability. Encapsulation is promising to improve the performance of medicines in oral health, such as mouthwashes.

2. Materials and Methods

2.1. Polymeric materials

The source of chitosan in this study was *X. gideon* obtained from Cangkurawok, Damaga, and Balumbang Jaya, Bogor, East Java. All parts of *X. gideon* body were detached, followed by drying for five days, and then continied to the processes of demineralization (3N HCl), deproteinization (3N NaOH), discoloration (4% H_2O_2) and deacetylation (50% NaOH) (Komariah et al. 2019).

2.2. Lemongrass extract (LE) preparation

Lemongrass (*Cymbopogon citratus*) was collected from Balai Penelitian Tanaman Rempah dan Obat (BALITRO), Indonesian Medicinal and Aromatic Crops Research Institute (IMACRI), West Java, Indonesia. The determination was carried out at Pusat Riset Biologi, Badan Riset dan Organisasi Nasional (BRIN), Cibinong, West Java, Indonesia. The leaves were dried in an oven at a temperature of 45 °C for one week and extracted using the maceration method (Felicia et al. 2022). They were soaked in 70% ethanol at a 1:10 (w/v) ratio for 24 h at room temperature. The macerated mixture was filtered using a filter paper and evaporated with a rotary vacuum evaporator at a temperature of 40 °C at 100 rpm for 2 h (Fitria et al. 2022).

2.3. The preparation of LE-loaded on chitosan X. gideon (LEChXg)

Chitosan with 83% degrees of deacetylation and a weight value of 0.5 g was dissolved in 1% acetic acid (Merck, Germany). Subsequently, 2 mL of 10% lemongrass leaf extract (LE) and 100 mL of distilled water were added (Veronica et al. 2021). Stirring was conducted using a magnetic stirrer (IKA RH basic 2, Germany) and heating at 40 °C with a speed of 2,500 rpm for 20 min and was subsequently carried out without heating for 100 min. Following the previous step, 40 mL of 0.1% tripolyphosphate (Sigma-Aldrich, USA) was added dropwise while stirring for one hour. Subsequently, 0.1 mL of 0.1% Tween 80 (Merck, France) was introduced, and the mixture was stirred again at a speed of 2,500 rpm for 30 min (Andikoputri et al. 2021; Veronica et al. 2021). The particle size of lemongrass extract was determined using a particle size analyzer (PSA) (Horiba Scientific, Nano Particle Analyzer SZ-100, UK) (Budi et al. 2020).

2.4. Culture of fibroblast

Human dermal fibroblast (HDF) was obtained from the Biorepository of Stem Cell Research Center, Yarsi University, Indonesia (Fitria et al. 2022). Fibroblast was planted in a cell culture dish and incubated at 37 °C with 5% CO_2 for 24 h. The growth medium was replaced by media containing DMEM, 10% FBS, and antibiotic-antimycotic (penicillin, streptomycin, and amphotericin B). The cultured cells were divided into eight treatment groups as follows: (1) without any treatment or stressor, (2) treated with hydrogen peroxide stressor (H_2O_2) as a negative con-

trol, (3) treated with H_2O_2 plus ascorbic acid as a positive control, (4–8) treated with H_2O_2 plus nanochitosan-encapsulated lemongrass (LEChXg) at concentrations of 100, 200, 300, 400, and 500 µg/mL, respectively. The stressor was given as the addition of 100 µM H_2O_2 (Lionetti et al. 2019) followed by incubation at 37 °C with 5% CO_2 for 60 min (Fitria et al. 2022).

2.5. Proliferation assay

Fibroblasts planted at a density of 1 × 10^3 cells/well in a 96-well plate were incubated at 37 ºC for 24 h. After 24, 48, and 72 h of treatment with a various concentration of LEChXg, the cells were washed by PBS 1×, and 100 µL of CCK-8 solution was added. The absorbance was measured at 450 nm using a microplate reader (Tecan Group Ltd. Mannedorf, Switzerland) and the percentage of fibroblast proliferation was calculated as shown below (Felicia et al. 2022).

$$proliferation\ rate\,(\%) = \frac{absorbance\ of\ sample}{absorbance\ of\ negative\ control} \times 100 \quad (1)$$

2.6. Migration assay

The cells were planted in a 24-well plate (2.9 × 10^3 cells/well) (Kauanova et al. 2021) and incubated until confluent. After reaching confluency, the monolayer was scratched gently using a white tip perpendicularly to the bottom of the monolayer. After the first scratch was conducted, a second scratch was made by crossing the first one. Thus, a cross pattern would be formed. Next, the cells were washed in PBS once and subsequently were treated (Felicia et al. 2022). The cell migration was observed after 0, 2, 4, 6, 24, and 48 h after the that and photographed using a microscope (EVOS FLc Cell Imaging System). At the end of the experiment, the wound closure was analyzed using ImageJ.

2.7. Data Analysis

Statistical quantification was conducted using SPSS version 2.3 and the data were presented as mean ± standard deviation (SD). Meanwhile, MANOVA was used to compare the groups against several dependent variables (times) in migration and proliferation assays. For a significant difference ($p < 0.05$), Post Hoc's Tukey test was performed.

3. Results and Discussion

3.1. Characteristics of LEChXg particle

Characteristic of LEChXg particle was used to estimate and determine the particle size and distribution of particle size. Meanwhile, particle size was measured using a PSA with a repetition of three times. The result of particle measurement and polydispersity index (PDI) LEChXg is shown in Table 1.

TABLE 1 Characterization of LEChXg nanoparticles.

Sample	Particle size (nm)	PDI	Zeta Potential (mV)
LEChXg	489.57 ± 3.44	0.69 ± 0.06	31.2 ± 0.87

PDI: poly dispersity index.

The LEChXg particle measurement indicated that the average size was 489.57 nm. Therefore, LEChXg fulfilled the requirements as a nanoparticle with a size range between 50–500 nm particle (Ismail and Harun 2019). According to Idacahyati et al. (2021), a nano-size particle should range from 1–1000 nm. PDI is a value that shows particle size distribution with a range of 0–1. A sample with a bigger and smaller size range has higher PDI values (Karmakar 2019). The result of particle distribution with PDI values 0.035 to 0.05 is considered to have monodisperse particle distribution (Clayton et al. 2016). Polydispersity is a macromolecule with various good weights, sizes, and mass distribution (Kim et al. 2019). The zeta potential value of LEChXg was 31.2 mV. Nanoparticles with zeta potential values smaller than 31.2 mV and greater than +30 mV also indicated good stability (Prakash et al. 2014). A dispersion system with a small zeta potential value was easier to form, such as the Van der Waals style in particle interaction (Juliantoni et al. 2020).

3.2. Migration of fibroblast

The migration test was conducted using scratch by making an artificial gap (scratch) in confluent monolayer cells. The gap allowed cells to communicate with each other. It also showed the ability of fibroblasts to move toward the ulcer, carry out proliferation, and form an extracellular matrix. The results of fibroblast migration showed that migration in 0 and 2 h did not indicate a significant difference ($p = 0.222$). However, at the observations of 4 and 6 h, there was a significant difference ($p < 0.05$) between the group with a stressor and the LEChXg group at a concentration of 300 µg/mL. The group treated with LEChXg showed a good migration activity by closing the most significant gap compared to those treated with a H_2O_2 stressor only. At 24 h, all study groups had no significant difference ($p > 0.05$). However, the fibroblast group treated with stressor showed less optimal migration due to an open gap of 0.51 ± 0.88 µm^2. The average fibroblast migration ability is shown in Table 2. Microscopic observation of the cell migration can be seen in Figure 1.

The observation of fibroblast migration when closing the gap is related to the ulcer healing process. The inflammation cells are stimulated to release various mediators (Chen et al. 2018) and growth factors such as the transforming growth factor-beta 1 (TGF-β) factor of fibroblast when oxidative stress is reduced (Jimi et al. 2020). H_2O_2 is one of the most critical compounds in ROS signaling studies due to its physicochemical properties, relatively low reactivity, and ability to diffuse through membranes. The addition at low concentrations increases intracellular ROS levels without causing oxidative stress. It also increases the migration of mesenchymal cells through extracellular signal-regulated kinases (ERK) 1/2 and focal adhesion kinase (FAK) pathways. Cellular abnormalities are increased when cells experience oxidative stress (Waheed

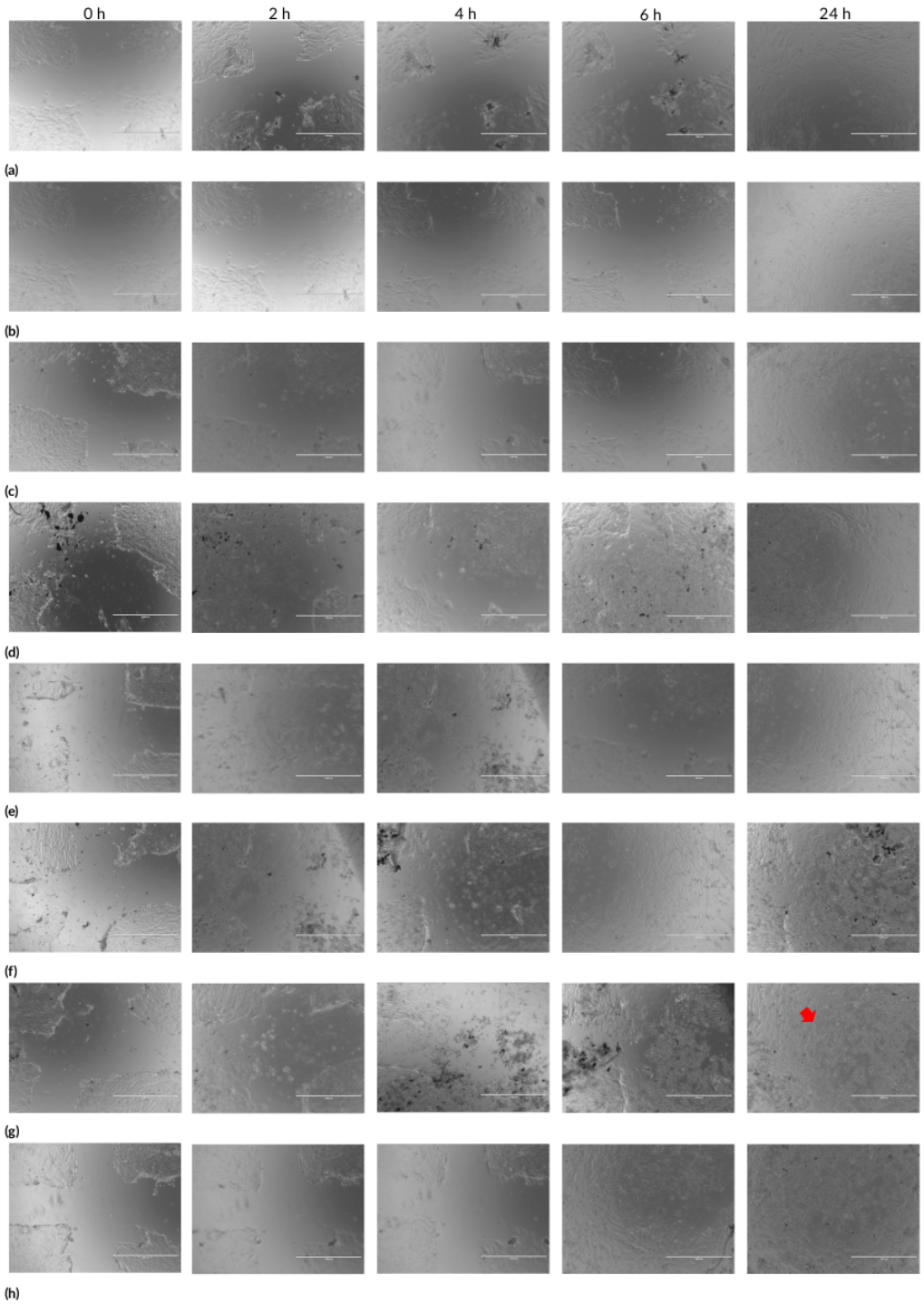

FIGURE 1 Fibroblast migration at 0, 2, 4, 6, and 24-h observation after the scratch wound. The yellow arrow shows a shaped artificial gap. The red arrow shows the artificial gap closing caused by the fibroblast migration. A: negative control, B: H_2O_2 only. C-H received H_2O_2 with treatment as follows: C: ascorbic acid as positive control, D: LEChXg 100 µg/mL, E: LEChXg 200 µg/mL, F. LEChXg 300 µg/mL, G. LEChXg 400 µg/mL, and H LEChXg 500 µg/mL. The observation was at 1,000× magnification. Scale bar = 1000µm.

TABLE 2 Fibroblast migration.

Group	Measurement number (n)	Migration (μm^2)				
		0 h	2 h	4 h	6 h	24 h
Untreated	3	2.87 ± 0.14	2.71 ± 0.28	2.53 ± 0.18^{ab}	2.50 ± 0.18^{ab}	0.00 ± 0.00
H_2O_2	3	3.82 ± 0.88	3.75 ± 0.91	3.41 ± 1.04^{a}	3.21 ± 1.04^{a}	0.51 ± 0.88
H_2O_2 + Ascorbic Acid	3	2.99 ± 0.20	2.82 ± 0.18	2.74 ± 0.19^{ab}	2.61 ± 0.17^{ab}	0.00 ± 0.00
H_2O_2 + LEChXg 100 µg/mL	3	2.70 ± 0.27	2.55 ± 0.28	2.24 ± 0.49^{ab}	1.88 ± 0.33^{ab}	0.00 ± 0.00
H_2O_2 + LEChXg 200 µg/mL	3	3.31 ± 0.05	2.89 ± 0.23	2.58 ± 0.18^{ab}	2.49 ± 0.18^{ab}	0.00 ± 0.00
H_2O_2 + LEChXg 300 µg/mL	3	2.92 ± 0.08	2.61 ± 0.18	1.44 ± 0.20^{b}	1.38 ± 0.16^{a}	0.00 ± 0.00
H_2O_2 + LEChXg 400 µg/mL	3	3.99 ± 1.73	3.43 ± 1.50	3.16 ± 1.38^{ab}	2.55 ± 1.11^{ab}	0.00 ± 0.00
H_2O_2 + LEChXg 500 µg/mL	3	2.68 ± 0.04	2.32 ± 0.16	2.03 ± 0.21^{ab}	1.94 ± 0.21^{ab}	0.00 ± 0.00

a-b in different columns migration shows a significant difference ($p < 0.05$), The superscript 'a' indicates a higher migration compared to group 'b,' while 'ab' indicates migration that is not different from groups 'a' and 'b'.

et al. 2022). Buranasin et al. (2018) stated that gingival fibroblast exposed to high glucose concentrations causes oxidative stress by increasing ROS production and inhibiting the migration process associated with inhibiting basic fibroblast growth factor (bFGF) signaling.

3.3. Fibroblast proliferation

The results of fibroblast proliferation in 24 h showed that the group treated with stressor was significantly different ($p < 0.05$) from those without stressor, as well group treated with ascorbic acid as a non-enzymatic antioxidant and LEChXg. The group treated with stressors indicated low cell proliferation compared to the others. Hydrogen peroxide is a molecule with low reactivity but can easily penetrate the cell membrane, generating the most reactive type of oxygen, hydroxyl radical, and converting Fe^{2+} atau Cu^{+} to OH (Nita and Grzybowski 2016).

Fibroblast proliferation without stressors had the highest proliferative compared to the other groups. The average proliferation is shown in Figure 2. Fibroblast can counteract an elevation in free radicals by augmenting the synthesis of endogenous antioxidants, thereby preventing any adverse impact on the proliferation of stressor-unexposed cells (Tsuneda 2020) The group treated with ascorbic acid showed good proliferation after exposing the cells to stressors H_2O_2. Ascorbic acid is an antioxidant that can neutralize oxidative stress by donating an electron to prevent other oxidized compounds and scavenging superoxide anion, hydroxyl radical, and lipid hydroperoxide (Pehlivan 2017).

Fibroblast proliferation in 48 h of observation showed that the group treated with H_2O_2 is significantly different ($p < 0.05$) from those without stressor, as well as the group treated with ascorbic acid, and LEChXg concentrations at 200, 300, and 400 µg/mL. Meanwhile, the group with stressors did not report a significant difference ($p > 0.05$) from LEChXg 100 and 500 µg/mL.

The group treated with H_2O_2 at 48 h showed a decrease in proliferation compared to the 24-h observation. It indicates that the cells experienced oxidative stress could not detoxify or repair the damage resulting from free radicals (Phaniendra et al. 2015). Therefore, it caused cell damage and affected proliferation. The ascorbic acid group and those treated with LEChXg at all the the tested concentra-

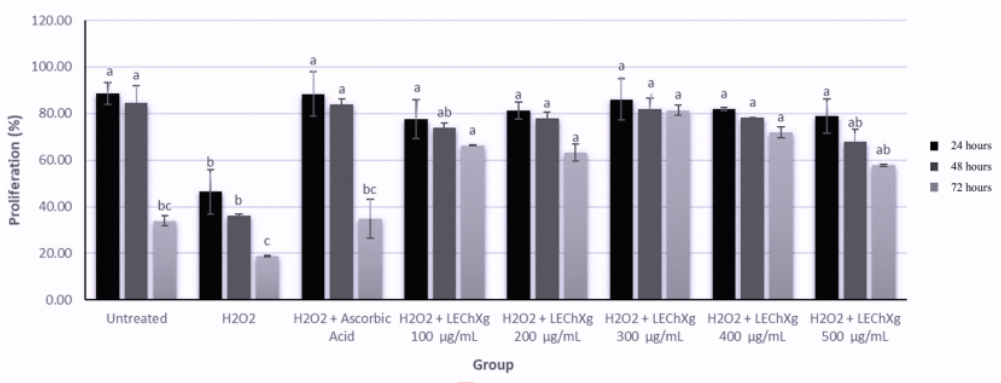

FIGURE 2 Fibroblast proliferation. a-c in different hours shows a significant difference ($p < 0.05$) The superscript 'a' indicates higher proliferation compared to groups 'b' and 'c,' while 'b' indicates higher proliferation than group 'c.' Superscript 'ab' implies no significant difference between groups 'a' and 'b,' and superscript 'bc' no significant difference between groups 'b' and 'c'.

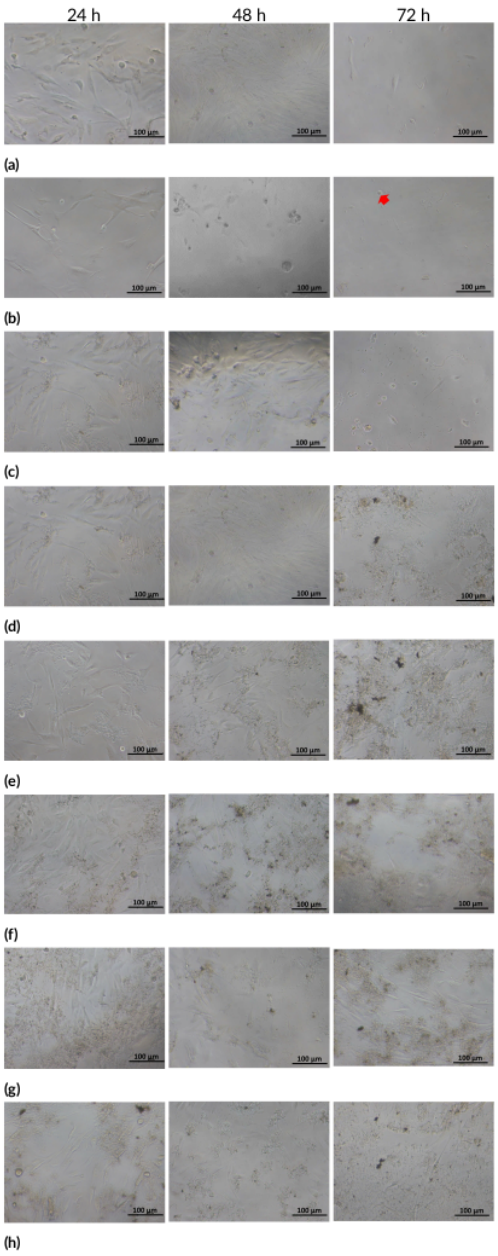

FIGURE 3 Fibroblast proliferation at 24, 48, and 72-h observation as indicated. The yellow arrow shows the fibroblast, which is living with a clearly visible cell nucleus, while the red arrow shows a dead fibroblast by shrinking in the cells. A: negative control, B: H_2O_2 only. C-H received H_2O_2 with treatment as follows: C: ascorbic acid as positive control, D: LEChXg 100 µg/mL, E: LEChXg 200 µg/mL, F. LEChXg 300 µg/mL, G. LEChXg 400 µg/mL, and H LEChXg 500 µg/mL. The observation was at 1,000× magnification.

tions could detoxify or repair the damages resulting from increased free radicals to enable a relatively stable proliferation.

The observation at 72 h showed that the group treated with stressor was significantly different ($p < 0.05$) from LEChXg concentrations 100, 200, 300, and 400 µg/mL. The difference showed increased proliferation higher than the group treated with a stressor. In contrast, the non-stressor, ascorbic acid, and LEChXg 500 µg/mL groups showed no significant difference ($p = 1.000$). Even though there was no difference, these groups exhibited higher proliferation cells than those treated with stressors. The proliferation of fibroblast in 24, 48, and 72 h observation is shown in Figure 3.

The results of the proliferation of the non-stressor, stressor, and ascorbic acid groups experienced decreased proliferation. However, a proliferation of the group treated with LEChXg was relatively stable. LEChXg maintained or stabilized proliferation until 72 h observation of fibroblast, which experienced oxidative stress. Pan et al. (2022) showed that active compounds of lemongrass increase cell proliferation power by reducing oxidative stress resulting from high ROS. Similarly, Roriz et al. (2014) indicated that lemongrass showed an antioxidant effect by improving the superoxide dismutase enzyme (SOD) activity and reducing the production of ROS in macrophages. Chitosan plays a role in scavenging free radicals and inhibiting oxidative damage (Pellis et al. 2022). The leading functional group, such as hydroxyl and an amino groups can reduce free radicals after a reaction at C-2, C-3, and C-6 positions of the pyranose ring to produce a stable macromolecule (Muthu et al. 2021). At the end of our observation, there was an aggregate formed in the LEChXg group due to polydisperse particle distribution with various molecular weights, sizes, and mass distributions, as well as zeta potential value which was relatively greater than +30 mV (Dipahayu and Kusumo 2021).

Our results were consistent with Beconcini et al. (2018), where the encapsulation of *Prunus avium* extract with chitosan and its derivatives reduces oxidative stress in human umbilical vein endothelial cells (HUVEC). An in vivo study on rat liver cells showed that encapsulated *Pinus merkusii* extract with chitosan reduces malondialdehyde (MDA) levels. An increase in MDA levels indicates hepatocyte cell membrane damage after exposure to ROS. Meanwhile, the decrease showed that the encapsulation of *P. merkusii* extract inhibits ROS production (Di Santo et al. 2021).

Damage in the epithelium and lamina propria as connective tissue with a predominance of fibroblast in mouth ulcers accelerated the healing process by encapsulating lemongrass leaf extract ethanol with chitosan *X. gideon*. The acceleration of the process was determined through increased migration and proliferation of fibroblasts after experiencing oxidative stress during the inflammatory process.

4. Conclusions

In conclusion, encapsulation of lemongrass leaf extract ethanol with chitosan *X. gideon* reduced fibroblast oxidative stress and was shown with good migration and proliferation at a range of 100–500 µg/mL, with the best concentrations at 300 µg/mL.

Acknowledgments

The authors are grateful to the Universitas Trisakti study institute for supporting the fund. The authors are also grateful to the Terpadu Laboratory in the Faculty of Medicine, Universitas Yarsi, BIOCORE and MICORE Laboratory in the Faculty of Dentistry, Universitas Trisakti, for the facilities provided.

Authors' contributions

KK designed the study, analysed the data, carried out the laboratory work and wrote the manuscript; PT wrote the manuscript; RW wrote the manuscript and analysed the data; NE carried out the laboratory work; DN wrote the manuscript. All authors read and approved the final version of the manuscript.

Competing interests

The authors declare no conflict of interest concerning the present paper.

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