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Research Article
Antioxidant and Anti-Inflammatory Activity of Ethanol Extract Stem of *Etlingera rubroloba* A.D. Poulsen

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Abstract

Background and Objective: *Etlingera rubroloba* A.D. Poulsen (*E. rubroloba*) is a plant endemic to South-East Sulawesi which is used empirically by local people as a pain reliever for joints and to increase endurance after typhoid fever. This study aimed to determine the antioxidant and anti-inflammatory activity of the ethanol extract stem of *E. rubroloba*. **Materials and Methods:** The stem of the ethanol extract of *E. rubroloba* with evaluated for antioxidants using the FRAP method and anti-inflammatory activity with the parameter level of Tumor Necrosis Factor-Alpha (TNF-α) *in vivo* by using 6 groups of rats, namely the normal, the negative (Na-CMC 0.5%), the positive control (Diclofenac sodium) and the ethanol extract group at doses of 200, 300 and 400 mg kg^{-1} b.wt. **Results:** The results of this study indicated that the antioxidant activity value of the IC_{50} ethanol extract was 12.720±0.12 μg mL^{-1} and ascorbic acid (vitamin C) as a standard control was 3.14±0.12 μg mL^{-1}. The TNF- α normal group (7.83 pg mL^{-1}), negative control (250.92 pg mL^{-1}), positive control (123.66 pg mL^{-1}), treatment group dose 200 (192.20 pg mL^{-1}), 300 (97, 95 pg mL^{-1}) and 400 mg kg^{-1} b.wt. (28.78 pg mL^{-1}). **Conclusion:** This study concluded that the ethanol extract of the stem of *E. rubroloba* has a very strong antioxidant activity and is an anti-inflammatory which is the best in reducing levels of TNF-α at a dose of 400 mg kg^{-1} b.wt.

Key words: *E. rubroloba*, ethanol extract, antioxidants, anti-inflammatory, Tumor Necrosis Factor-Alpha (TNF-α), FRAP method, *in vivo*

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Inflammation is a normal protective response caused by injury or tissue damage caused by physical trauma, damaging chemicals or the invasion of pathogenic microorganisms. There are two phases of inflammation, namely acute and chronic. Acute inflammation is the initial response to tissue injury that triggers local vasodilation and increases capillary permeability resulting in fluid accumulation in the injured area. Various mediators contribute to the inflammatory process such as serotonin, histamine, leukotrienes, prostaglandins and pro-inflammatory cytokines such as interferon (IFN), interleukin 1β and Tumor Necrosis Factor-Alpha (TNF-α)[1].

Cytokines are inflammatory mediators that function in the mobilization of other leukocytes to the injured tissue. The more neutrophils and macrophages that carry out phagocytosis, the more cytokines will be produced. Tumour Necrosis Factor Alpha (TNF-α) is a major cytokine in the acute inflammatory response. TNF-α will function as a protein that gives a signal if there is interference due to infection. Severe infections can trigger the production of large amounts of TNF-α causing systemic reactions[2].

Changes in environmental conditions and unhealthy lifestyles can make the body susceptible to various types of diseases. One of the causes of disease in the body is free radicals. To protect the body from free radical attack, we need a material that functions as an antioxidant. Antioxidants are secondary metabolites that are used to prevent free radicals. The higher the antioxidant activity, the more free radicals are prevented[3].

One of the plants studied is *Etlingera rubroloba* A.D. Poulsen from the genus Etlingera, this plant is thought to have various pharmacological activities. Etlingera species exist in the world about 150-200 species of which about 54 are found in Indonesia, including 48 species on the island of Sulawesi and 6 species from the island of Java. Species of this plant have been used in traditional medicine to treat various diseases and the presence of volatile and nonvolatile entities in these species is gaining research interest among scientists[9].

Various species of Etlingera that have been reported related to biological and pharmacological aspects are *E. elatior* as hepatoprotective and phytochemical screening results contain alkaloids, flavonoids, tannins and terpenoids[4]. *Etlingera calophrys* contains Yakuchinone A, p-Hydroxybenzoic acid and stigmasterol compounds and is active as an antfioxidant[5]. On the island of Sulawesi, the fruit of *E. elatior* is used as a medicine for nausea and treating typhoid ever[6]. *E. elatior* is also active as an antibacterial antioxidant[7,8]. *E. elatior* fruit is active as Antihyperuricemia[9]. Rhizome of *E. elatior* as antioxidant and antibacterial[7,10]. *E. elatior* is active as an antibacterial, deodorant, wound medicine, tyrosinase inhibitor and antioxidant[11]. *E. elatior* flower is a fungal antioxidant and antibacterial[12]. *E. coccinea* is an antioxidant in the rhizome, stem and leaves[13]. Meanwhile, *E. rubrostriata, E. Littoralis* and *E. Fulgens* are active as antibacterial and antioxidants[14]. *E. sessilanthera* and *E. coccinea* as antibacterial[15]. *E. pubescens* as antibacterial[16,17], flower extract of *E. elatior* as antihyperuricemia, antioxidant and chemotherapy against melanoma[18,19]. *Etlingera rubroloba* fruit as immunomodulator[20], fruit *E. rubroloba* has Immunomodulatory Potential on Diabetic-Infected Tuberculosis[21], *E. rubrolaba* stem methanol extract as antioxidant[22] and Xanthine Oxidase inhibitor[23]. *E. brevilabrum* as cholesterol-lowering[24]. *E. rubroloba* fruit as Immunostimulator with increased CD8[25].

This study was conducted to examine the potential of ethanol extract of *E. rubroloba* A.D. Poulsen as an antioxidant and anti-inflammatory with parameters of TNF-α levels. The results of this study can be developed as traditional medicine.

MATERIALS AND METHODS

Study area: This research was conducted in the laboratory of the UHO Faculty of Pharmacy, the Laboratory of the UHO Faculty of Medicine and the Faculty of Medicine, Universitas Brawijaya Malang Indonesia, from January to April, 2022.

Materials: The materials used were *E. rubroloba* stem, aqua dest (WaterOne®), 96% ethanol, filter paper, carrageenan, Aqua Pro Injection (Otsuka®), diclofenac sodium 50 mg, Na. CMC 0.5%, Potassium Ferricyanide ($K_3Fe(CN)_6$, $FeCl_3$, NaOH, KH_2PO_4, TCA, oxalic acid, pure vitamin C, ethanol pa, 1 cc and 3 cc syringe (OneMed®), microplate flat-bottom polystyrene 96 well (Iwaki, Japan), 1.5 mL Eppendorf tube (Onemed®), Rat TNF-α ELISA KIT. Eppendorf tube (Onemed®, Germany), Rat TNF-α ELISA KIT (Shanghai Korain Biotech Co., Ltd.).

Methods

Sample preparation and extraction: A total of 20 kg of stems of *E. rubroloba* A.D. Poulsen were collected from Laiwoi Village, Laeya District, Konawe Selatan Regency. The sample was determined at the Research Center for Biology, LIPI, Cibinong, Bogor, Indonesia. The samples were cleaned, dried under direct sunlight and then powdered. Then extracted with 96% ethanol (12 L, 3×24 hrs), using the maceration method. The obtained filtrate was evaporated with an evaporator (50°C) and a thick extract was obtained[26].

Antioxidant activity test FRAP method

Activity measurement: The stock solution of extract and vitamin C samples with five concentration variants, namely 25, 20, 15, 10 and 5 μg mL^{-1} 1 mL of each concentration, was taken, then 1 mL of 0.2 M phosphate buffer and 1 mL of 1% $K_3Fe(CN)$ 6 were added, then incubated. Then 1 mL of 10% TCA was added with the aim that the potassium ferricyanide complex precipitated, then centrifuged at 3000 rpm for 10 min to speed up the precipitation process. After that, 1 mL of the top layer was taken and put in a test tube, adding 1 mL of distilled water and 0.5 mL of 0.1% $FeCl_3$. The solution was allowed to stand for a few minutes and its absorption was measured with an ultraviolet-visible (UV-Vis) spectrophotometer at the maximum wavelength obtained.

Inhibition percentage: The antioxidant activity of the sample is determined by the amount of free radical uptake FRAP by calculating the percentage of solution absorption inhibition using the formula[27]:

$$\text{Inhibition (\%)} = \frac{\text{Blanko absorbance} - \text{Sample absorbance}}{\text{Blanko absorbance}} \times 100$$

The IC_{50} value was obtained from the % inhibition and concentration of the extract of *E. rubroloba* A.D. Poulsen by plotting the calculated values in a linear regression equation with concentration (ppm) as the X-axis and the percentage of inhibition as the Y-axis, so that the Eq:

$$Y = aX+b$$

Where:
Y = IC_{50} value
X = Sample
a = Slope/gradient
b = Intercept

Antioxidant activity is expressed by the IC_{50} value (50% Inhibition Concentration), which is the concentration of the sample that can reduce 50% FRAP radicals[27].

Anti-inflammatory activity test

Grouping of test animals: The test animals were divided into 6 test groups and the grouping of test animals was carried out entirely randomly with the number following the Federer formula. This study used a sample of 4 rats for each group and as a backup, if there was a sample failure in the study. So, the total number of samples used in this study was 30 individuals in 6 treatment groups.

Induction of inflammation: Edema was made on the right hind paw of rats by inducing 0.1 mL of 1% carrageenan solution subplantar. Carrageenan was chosen as an irritant for making edema because it has several advantages. Namely, it does not leave scars, does not cause tissue damage and can provide a more sensitive response to anti-inflammatory drugs than other irritants, so it is suitable to be chosen as an inductor for edema[28].

Inflammation measurement: Inflammation was measured by dipping the mice's feet into a tube containing the measuring solution to the mark. The change in the volume of the solution was recorded for a certain time (Vt) of the mice's feet. Inflammation volume is the difference in the volume of the mice's feet at a certain time (Vt) with the initial leg volume (Vo).

Anti-inflammatory potency test based on TNF-α

Treatment and collection and storage of rat blood: After the rats were induced with 1% carrageenan and experienced edema after 1 hr, the rats were given treatment in the form of administration of *E. rubroloba* stem ethanol extract 200, 300 and 400 mg kg^{-1} b.wt., as the test group, administration of Na-CMC 0 and 5% as a negative control and administration of sodium diclofenac as a positive control with a dose of 1.13 mg. After that, blood samples were taken in the second hour to assess the levels of the inflammatory mediator TNF-α. Blood sampling was carried out on the hearts of rats. After taking the blood, the blood is put in a tube containing the anticoagulant EDTA.

ELISA test: Enzyme-Linked Immunosorbent Assay (ELISA) for measuring levels of TNF-α follows protocol Rat Tumor Necrosis Factor, TNF-α ELISA Kit which measured absorbance at a wavelength of 450 nm.

Data analysis: The data obtained were analyzed statistically using the SPSS program. The effect of the ethanol extract of *E. rubroloba* stems and the decrease in TNF-α levels in all treatment and comparison groups was analyzed by using the One-way ANOVA (Analysis of Variance) statistical test. Then proceed with the *post hoc* Tests to compare between groups.

RESULTS AND DISCUSSION

Antioxidant activity of FRAP method: Measurement of antioxidant activity was carried out using the FRAP test, where the ethanol extract of the stem of *E. rubroloba* was used as a

sample and vitamin C as a standard solution. Antioxidant testing is expressed by the IC_{50} (inhibition concentration) parameter. The amount of antioxidant activity is indicated by the IC_{50} value, which is the concentration of the sample solution required to inhibit 50% of free radicals. The smaller the IC_{50} value of the compound, the greater the ability of the compound to ward off free radicals.

The result of determining the maximum wavelength of the FRAP solution is 582 nm with an absorbance of 0.576 nm. Determination of the antioxidant activity of the ethanolic stem extract of *E. rubroloba* A.D. Poulsen compared with ascorbic acid (vitamin C) standard[29].

The measurement results of antioxidant activity indicated that the comparison of vitamin C and the ethanol extract of stem *E. rubroloba* A.D. Poulsen has antioxidant activity values in the very strong category. The result of percentage inhibition in ascorbic acid has a higher percentage (%) of inhibition than the ethanol extract of E. rubroloba stem A.D. Poulsen, which indicates that the greater the percentage (%) of inhibition, the smaller the IC_{50} value, which indicates the greater the ability of the sample to capture free radicals. Thus, vitamin C used as a comparison has a stronger free radical scavenging ability than the ethanol extract of *E. rubroloba* A.D. Poulsen stem. Judging from the IC_{50} value, it is still a very strong category.

The results showed that the IC_{50} value of the *E. rubroloba* stem ethanol extract of A.D. Poulsen was 12.71±0.12 μg mL^{-1} and vitamin C was 3.14±0.12 μg mL^{-1}. The amount of antioxidant activity was indicated by the IC_{50} value, which is the concentration of the sample solution needed to reduce 50% of free radicals. A compound is said to be a very strong antioxidant if it has an IC_{50} value of less than 50 μg mL^{-1}, strong for an IC_{50} value of 50-100 μg mL^{-1} and moderate if it has a value of 101-250 μg mL^{-1}, weak if the IC_{50} value is 251-500 μg mL^{-1} and >500 have no antioxidant activity[30]. Based on these results, the IC_{50} value of Vitamin C and the ethanol extract of *E. rubroloba* stem A.D. Poulsen had an IC_{50} value of less than 50 μg mL^{-1}. Thus, the stem of *E. rubroloba* A.D. Poulsen is a plant with a very strong antioxidant activity. This study is in line with previous research, where the antioxidant test used methanol extract of *E. rubroloba* with the DPPH method with a very strong antioxidant value[22,23]. In addition, it was previously reported that a different species, *E. calophrys*, has a very strong antioxidant capacity[5].

Anti-inflammatory activity

Rat edema examination results: Edema is a buildup of fluid in the lower layers of the skin, which is a sign of inflammation. Carrageenan is a strong chemical substance that releases inflammatory and pro-inflammatory mediators (prostaglandins, leukotrienes, histamine, bradykinin, TNF-α, etc.). Carrageenan was chosen to test anti-inflammatory drugs because it is not antigenic and does not cause systemic effects. Cardinal signs of inflammation, namely edema, hyperalgesia and erythema, occur after injection due to the activity of pro-inflammatory agents, such as bradykinin, histamine, complement and reactive oxygen[31]. Neutrophils immediately migrate to sites of inflammation and can produce pro-inflammatory reactive oxygen species. Based on the plethysmometer test, the average volume measurement results for each treatment group were obtained, as shown in Fig 1.

The samples used were the feet of rats that had edema after being induced by carrageenan with a concentration of 1% and a decrease in the volume of the rat's feet after administration of the test solution. The anti-inflammatory activity of the test material was shown by its ability to reduce edema caused by carrageenan induction in the soles of mice. The research was carried out by injecting 1% carrageenan suspension into the paws of the male Wistar strain test rats intraplantar and then giving the test solution.

Results of examination of TNF-α levels in Wistar strain male rats: Measurement of TNF-α levels using Enzyme-Linked Immunosorbent Assay (ELISA). The ELISA was performed to see the quantitative or qualitative TNF-α based on colorimetric readings[32]. Measurement of TNF-α levels was carried out by taking rat blood 1 hr after carrageenan induction. This blood collection time was adjusted to the peak level of TNF-α as a pro-inflammatory cytokine in the 1st hr. The levels obtained will be the initial data on TNF-α levels, namely when inflammation occurs. After that, each group was given their respective treatment. One hour later, blood was drawn and centrifuged to obtain plasma. This plasma is then read on the ELISA reader. The results of the TNF-α levels can be seen in Fig. 2.

Based on Fig. 2, It can be seen that the normal group is the group that has the lowest levels, namely 7.83 pg mL^{-1}, because it is the level of TNF-α in the physiological state of the body. Where TNF-α will increase as the degree of inflammation increases. Inflammation is the body's defense response against the entry of foreign substances in the form of carrageenan so that pro-inflammatory cytokines such as TNF-α appear as an immune response. The TNF-α acts as an immune response modulator that can mediate the induction of adhesion molecules. Increased induction of adhesion molecules serves to facilitate leukocytes' adherence to the endothelium surface and activate leukocytes. These

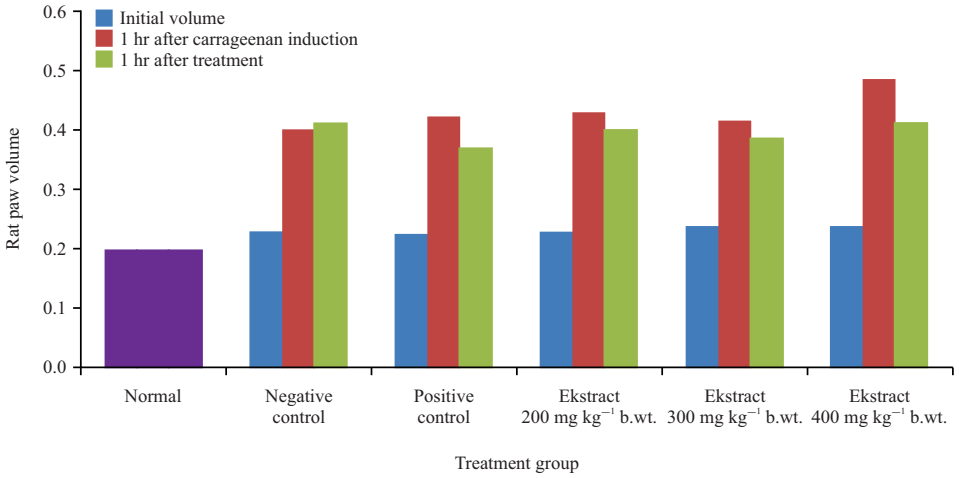

Fig. 1: Volume of edema before and after treatment

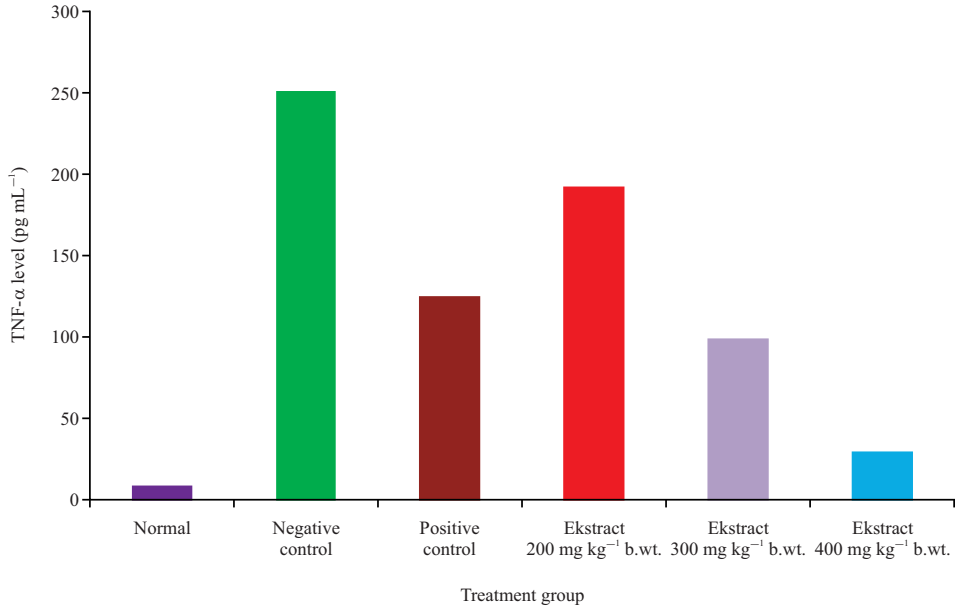

Fig. 2: TNF-α levels in the treatment and normal group

leukocytes will neutralize inflammatory agents at the site of injury, so the higher the degree of inflammation, the more leukocytes needed and the more TNF-α levels must play a role.

These data indicated that the administration of negative Na-CMC control was the initial comparison of the entire treatment group. The level of negative control was 250.92 pg mL^{-1}. This value is not much different from the decrease in levels caused by the administration of the ethanol extract of stem *E. rubroloba* A.D. Poulsen 200 mg kg^{-1} b.wt., equal to 192,20 pg mL^{-1}. This indicated that the extract with a dose of 200 mg kg^{-1} b.wt., has less effect as an anti-inflammatory. However, the decrease in TNF-α levels occurred as the dose of the extract was increased. In the extract with a dose of 300 mg kg^{-1} b.wt., the levels of TNF-α decreased to 97.95 pg mL^{-1}, where this value is lower than the dose of 200 mg kg^{-1} b.wt. This shows that a 300 mg kg^{-1} b.wt., dose has a better anti-inflammatory effect than 200 mg kg^{-1} b.wt. At the same time, the lowest value of all dose variations is 400 mg kg^{-1} b.wt., which can reduce TNF-α levels to 28,78 pg mL^{-1}. This showed that a 400 mg kg^{-1} b.wt., dose has a better anti-inflammatory effect than a dose of 200 and 300 mg kg^{-1} b.wt. and positive control (123.66 pg mL^{-1}). The

anti-inflammatory activity of the ethanolic stem extract of *E. rubroloba* A.D. Poulsen was probably due to the presence of flavonoids, which can decrease the secretion of pro-inflammatory cytokines such as TNF-α[33].

The data obtained were carried out by the Shapiro-Wilk Test to see the normality of the data and the Levene test to see the homogeneity of the data. Based on the normality test of the data using the Shapiro-Wilk test, it is known that the data distribution of all groups is normally distributed ($p>0.05$) and based on the results of the data variance test that the data has the same or homogeneous variance ($p>0.05$). Therefore, the data were analyzed by the One-way ANOVA (Analysis of Variance) Test. Based on the One-way ANOVA Test, the value ($p<0.05$) was obtained so that it could be interpreted that there was a significant effect of the administration of ethanol extract of *E. rubroloba* A.D. Poulsen stem on TNF-α levels in male Wistar rats after the 3 hrs of treatment.

CONCLUSION

The ethanol extract of stem *E. rubroloba* A.D. Poulsen has antioxidant activity with a very strong category and has potential as an anti-inflammatory by TNF-α parameters and this research is a reference in the development of traditional medicines. The conclusion of this study showed that the ethanol extract of the stem *Etlingera rubroloba* A.D. Poulsen had a very strong category of antioxidant activity with an IC_{50} value of 12.71 ± 0.12 g mL^{-1} as measured using the FRAP method and had acted as an anti-inflammatory with *in vivo* by reducing TNF-α levels at a dose of the best is 400 mg kg^{-1} b.wt. This research is a reference in the development of traditional medicine, especially gout, rheumatism, uric acid and natural antioxidants.

SIGNIFICANCE STATEMENT

This study found that the stem of *Etlingera rubroloba* A.D. Poulsen has very strong antioxidant and anti-inflammatory activity with the parameter of Tumor Necrosis Factor-Alpha (TNF-α). This research is a reference for the development of traditional medicine.

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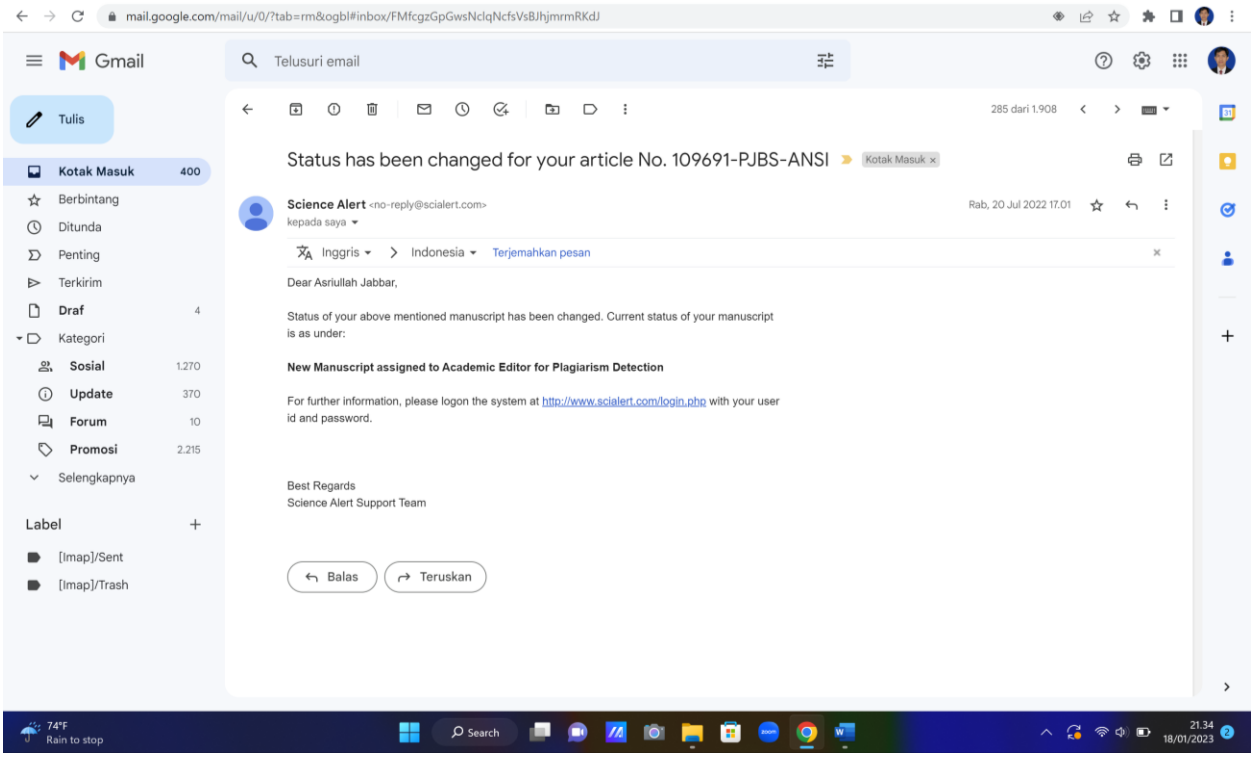

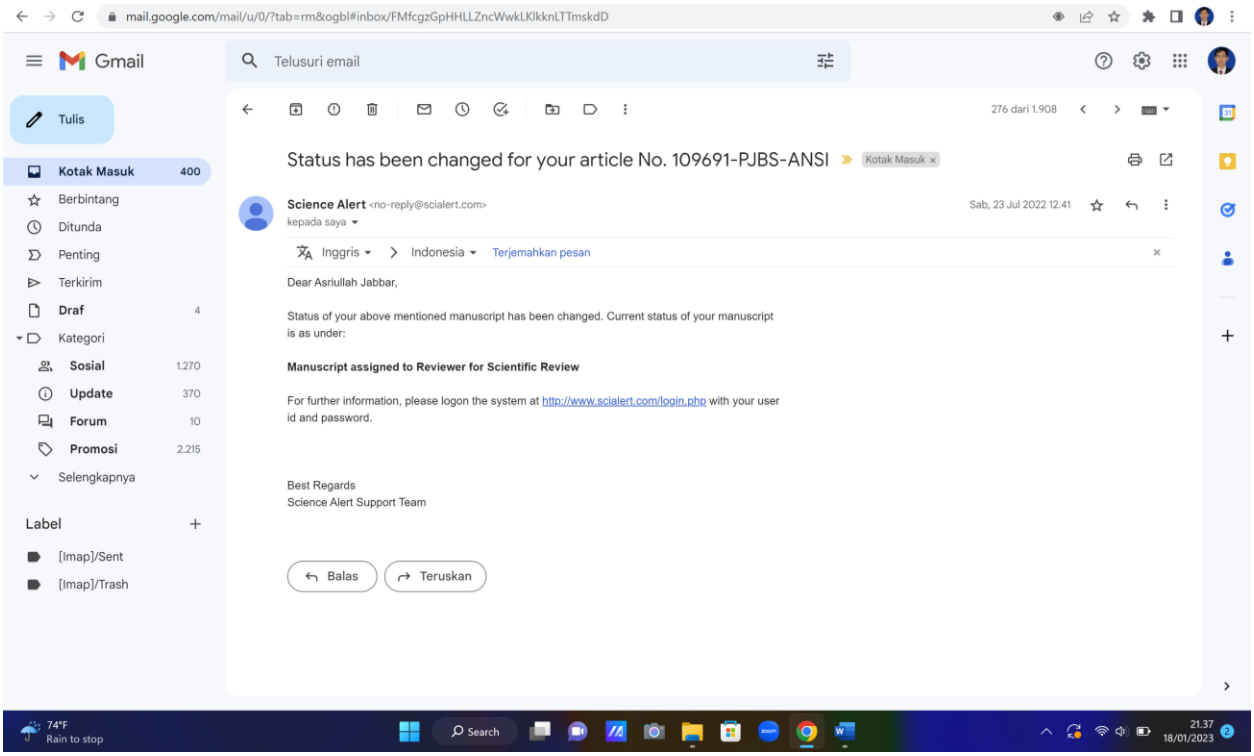

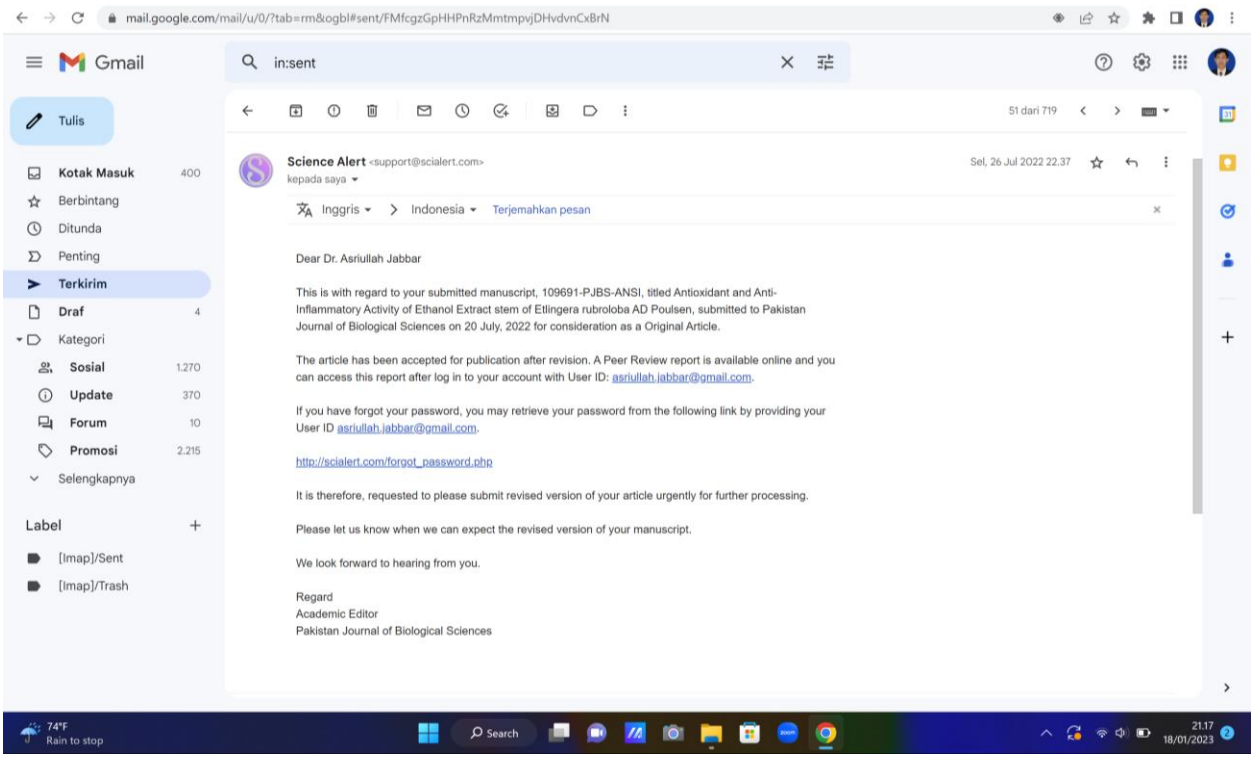

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This is with regard to your submitted manuscript, 109691-PJBS-ANSI, titled Antioxidant and Anti-Inflammatory Activity of Ethanol Extract stem of Etlingera rubroloba AD Poulsen, submitted to Pakistan Journal of Biological Sciences on 20 July, 2022 for consideration as a Original Article.

The article has been accepted for publication after revision. A Peer Review report is available online and you can access this report after log in to your account with User ID: asriullah.jabbar@gmail.com.

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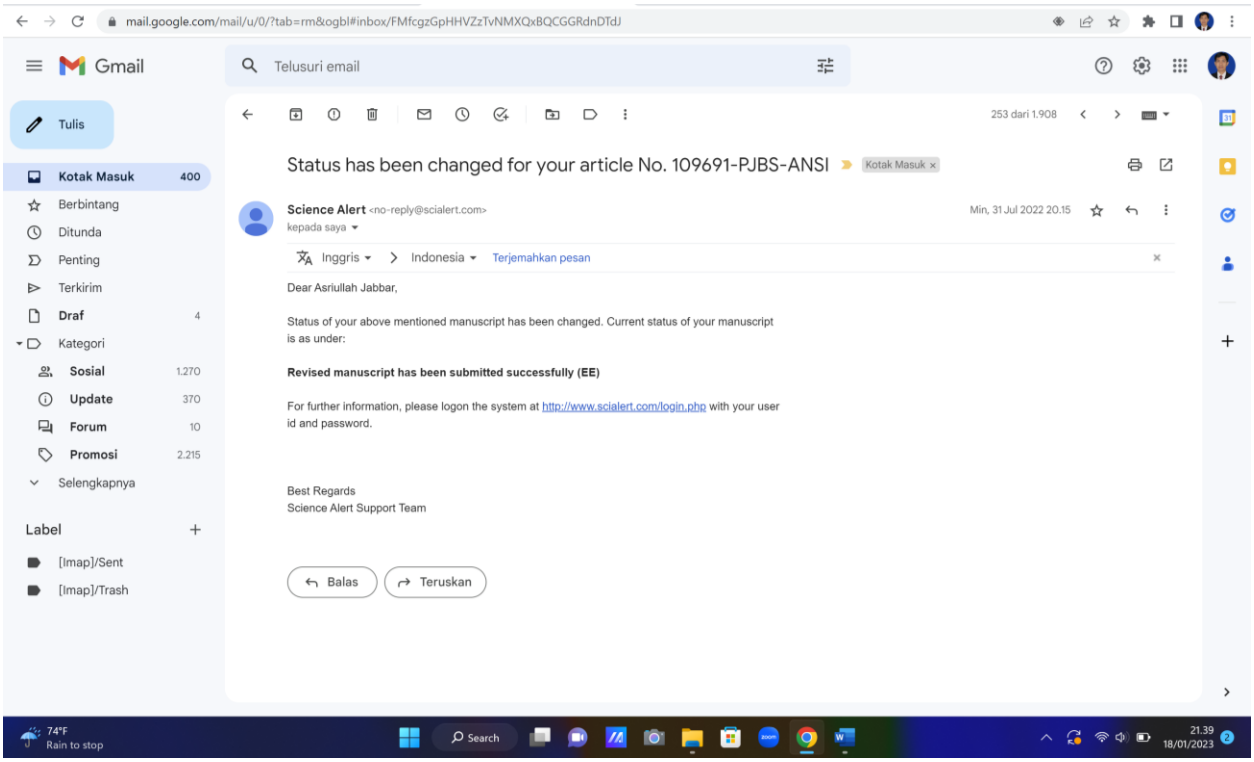

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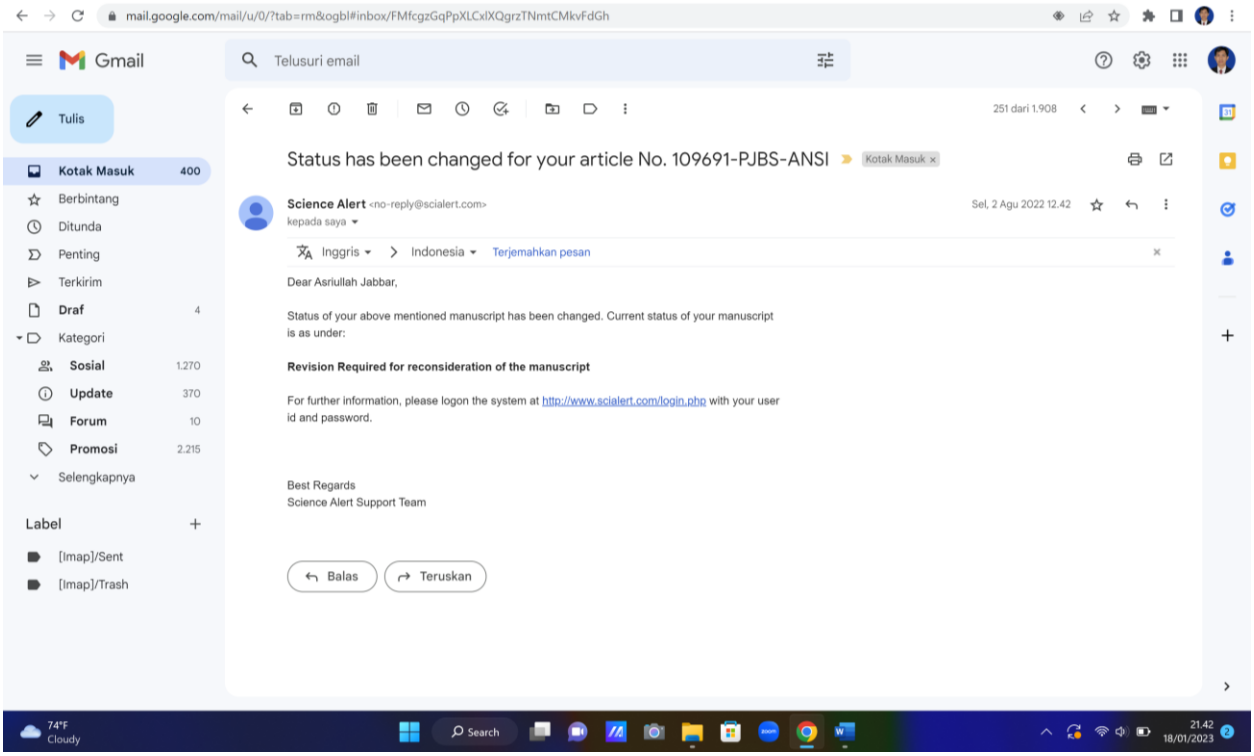

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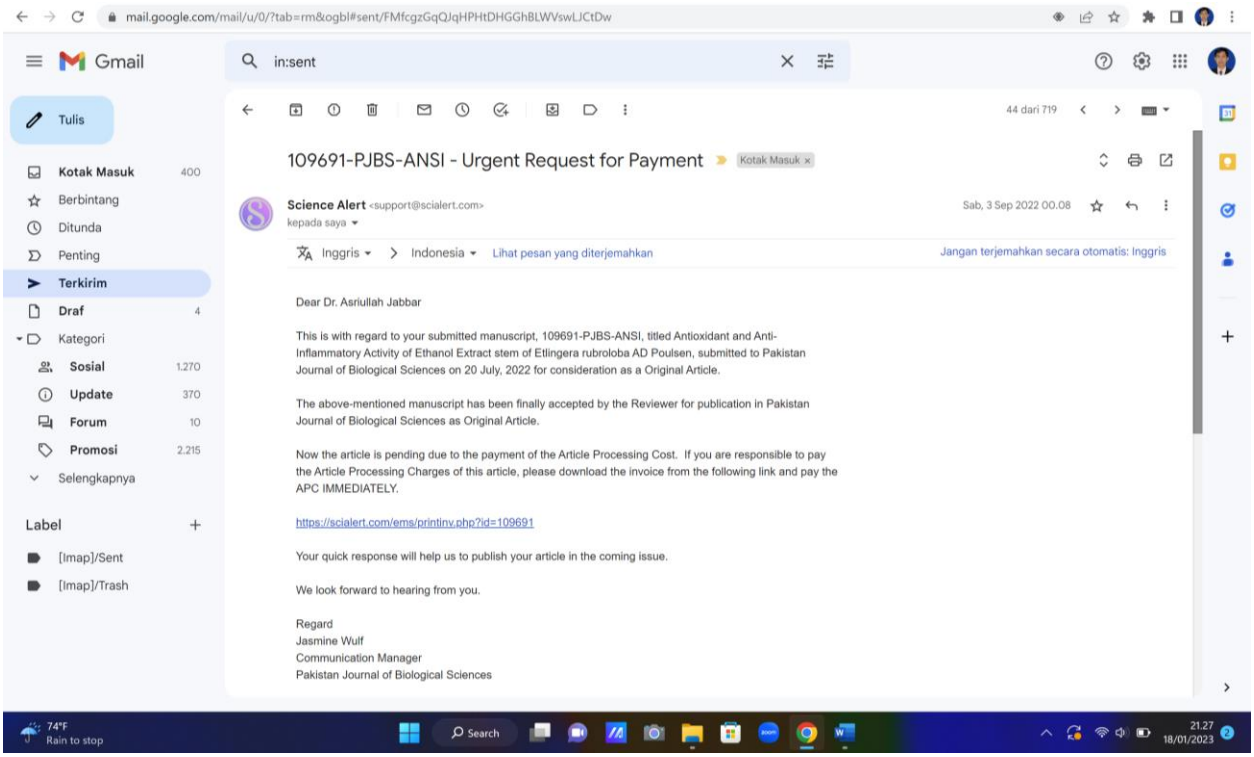

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Dear Dr. Asriullah Jabbar

This is with regard to your submitted manuscript, 109691-PJBS-ANSI, titled Antioxidant and Anti-Inflammatory Activity of Ethanol Extract stem of Etlingera rubroloba AD Poulsen, submitted to Pakistan Journal of Biological Sciences on 20 July, 2022 for consideration as a Original Article.

The above-mentioned manuscript has been finally accepted by the Reviewer for publication in Pakistan Journal of Biological Sciences as Original Article.

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Surat Kelaikan Etik (*Ethical Clearance)*
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KELAIKAN ETIK (*ETHICAL CLEARANCE*)
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Yang bertanda tangan dibawah ini, Ketua Komisi Etik Penelitian kesehatan Lembaga Penelitian dan Pengabdian pada Masyarakat Universitas Halu Oleo, setelah dilaksanakan pembahasan dan penilaian, dengan ini memutuskan protokol penelitian yang berjudul:

"Antioxidant and Anti-Inflammatory Activity of Ethanol Extract Stem of Etlingera rubroloba A.D. Poulsen"

yang mengikutsertakan hewan coba sebagai subjek penelitian, dengan:

Ketua Pelaksana/Peneliti Utama	:	Dr. Asriullah Jabbar
Unit/lembaga	:	Fakultas Farmasi Universitas Halu Oleo
Tempat penelitian	:	Laboratorium Hewan Coba Fakultas kedokteran UHO dan Laboratorium Farmakologi Fakultas Farmasi UHO

dapat disetujui pelaksanaannya. Persetujuan ini berlaku sejak tanggal ditetapkan sampai dengan batas waktu pelaksanaan penelitian seperti tertera dalam protokol.

Pada akhir penelitian, laporan pelaksanaan penelitian harus diserahkan kepada KEP-LPPM UHO. Jika ada perubahan protokol dan/atau perpanjangan penelitian, harus mengajukan kembali permohonan kajian etik penelitian (amandemen protokol).

Kendari, 20 April 2022

Ketua
Komisi Etik Penelitian
LPPM UHO,

Dr. Nani Sunarti, M.Kes