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Suppression of pro-inflammatory cytokines and mediators production by ginger (*Zingiber officinale* Roscoe) ethanolic extract and gingerol in lipopolysaccharide-induced RAW 264.7 murine macrophage cells

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Chronic inflammation could lead to several life-threatening diseases such as cancer and cardiovascular diseases. Ginger (*Zingiber officinale* Roscoe) has been used for many years to treat various diseases and health problems, including inflammation. This study was conducted to assess ginger ethanolic extract (GEE) and its compound gingerol's potential as an anti-inflammatory agent by evaluating the concentration of pro-inflammatory cytokines and mediators such as TNF-α, IL-1β, IL-6, COX-2, and NO in LPS-induced RAW 264.7 cells. The safe concentration of GEE and gingerol for the RAW 264.7 cells were evaluated using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay. The quantification of TNF-α, IL-1β, IL-6, COX-2, was conducted based on the ELISA method, while the quantification of NO was conducted by the nitrate/nitrite colourimetric method. The results showed that GEE and gingerol were able to inhibit TNF-α, IL-1β, IL-6, COX-2, and NO production in LPS-induced RAW 264.7 cells. GEE has better anti-inflammatory activity than gingerol. GEE in the concentration of 50 µg/mL has the highest inhibition activity over positive control or inflammatory cells model. GEE exhibited good anti-inflammatory properties through reduction of pro-inflammatory mediators such as TNF-α, IL-1β, IL-6, COX-2 and NO. Thus, ginger ethanolic extract has a high potential in the treatment of inflammation-related diseases.

Keywords: Cytokines, Gingerol, Inflammation, Macrophage, *Zingiber officinale*.

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Introduction

Plants have long over the years been used as a popular mode of therapies for the treatment of numerous health problems[1]. One of the plants known to possess therapeutic properties is ginger (*Zingiber officinale* Roscoe), which also usually used as a spice in foods[2,3]. As a traditional herbal medicine, ginger is usually used to treat arthritis, rheumatism, sprains, muscular aches, pains, sore throats, cramps, constipation, indigestion, vomiting, hypertension, dementia, fever, infectious diseases, and helminthiasis[3]. The plant contains several active phenolic compounds such as gingerol, paradol, and shogoal proven to have antioxidant, anti-cancer, anti-angiogenesis, and anti-atherosclerotic activities[2]. The anti-inflammatory potential of ginger has also been reported by several studies[4].

Chronic inflammation has been popularly known to be linked with various diseases such as cardiovascular diseases, diabetes, arthritis, Alzheimer's disease, pulmonary diseases, cancer, and autoimmune diseases[2]. Inflammation is an innate response towards irritation and infection caused by pathogens, wounds, and chemicals, it is a complex process regulated by pro-inflammatory cytokines and mediators[5]. Macrophage, which is the cell line of chronic inflammation, is activated during inflammation by the exposure to interferon-γ (IFN-γ), pro-inflammatory cytokines, or bacterial lipopolysaccharides (LPS)[6-9]. The activated macrophage release several chemicals including reactive oxygen species (ROS), Nitric Oxide (NO), prostaglandin E (PGE), and cytokines (Interleukin-1β, Interleukin-6, and Tumor Necrosis Factor-α (IL-1β, IL-6, TNF-α) and cyclooxygenase-2 (COX-2)[7-9]. Overproduction of inflammatory mediators and cytokines in prolonged inflammation can cause cellular and tissue damages that lead to several diseases, therefore anti-inflammatory agents are important to prevent the side effect of prolonged inflammation and suppress the production of inflammatory marker molecules[8,9].

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In this study, the anti-inflammatory properties of ginger ethanolic extract (GEE) along with its compound gingerol assessed in LPS-induced RAW 264.7 murine macrophage cells as inflammation model by evaluating the TNF-α, IL-1β, IL-6, COX-2, and NO levels in the inflammatory cells model.

Materials and Methods

Plant extract preparation

The ginger (*Z. officinale* Roscoe) were collected from farmer plantation located in Bogor, West Java. The plant was identified by the staff of Herbarium, Department of Biology, School of Life Sciences and Technology, Bandung, West Java, Indonesia. The rhizome was chopped and dried using food dehydrator (40-45 ^{o}C) until stable water with level (±13%) was achieved. The dried rhizome of ginger was mashed and crushed into small pieces. Briefly, 200 mg of ginger powder was extracted by maceration technique with 70% ethanol solvent. Every 24 hours, the filtrate was collected until ethanol filtrate turned colourless. The filtrate was then evaporated using a rotary vacuum evaporator (Zhengzhou RE-201D) at a temperature 50 ^{o}C until extract was obtained in paste form. The paste product, which was ginger ethanolic extract (GEE) was stored at -20 ^{o}C for further use[9-14]. The gingerol (BP0092) were purchased from Chengdu Biopurify Phytochemical Ltd.

RAW 264.7 cells culture

The RAW 264.7 (ATCC®TIB-71™) murine macrophage cell line was obtained from Biomolecular and Biomedical Research Center, Aretha Medika Utama. The cells were grown in Dulbecco's Modified Eagle Medium (DMEM) (Biowest, L0104) supplemented with 20% Fetal Bovine Serum (FBS) (Biowest, S1810), 1% Penicillin-Streptomycin (Penstrep) (Biowest, L0022). The culture was incubated at 37 °C with 5% CO_2 in a humidified atmosphere until the cells were confluent (80-90%). The confluent cells then harvested using trypsin-EDTA (Biowest, X0930) and seeded on plates for the next assays[11,12].

Viability assay of RAW 264.7 cells toward GEE and gingerol

The determination of the number of viable cells was performed using MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay (Promega, G3582). Around 5 x 10^3 cells in 100 µL of medium (DMEM supplemented with 10% FBS and 1% Penstrep were plated on each well in 96-well plate, incubated for 24 hours at 37 ^{o}C, 5% CO_2, humidified atmosphere. The prior medium then washed and the cells supplemented with 90 µL of fresh medium and 10 µL of GEE (100, 50, 10 µg/mL) and gingerol (100, 50, 10 µM). After 24 hours, each well was added with 20 µL of MTS, incubated at 37 ^{o}C, 5% CO_2 for 3 hours. The absorbance was then measured at 490 nm with a spectrophotometer (Multiskan™ GO and Microplate Spectrophotometer, Thermo Scientific N12391). The untreated cells served as a control and the viability percentage was calculated from the viable cells from each treatment toward the control. The concentration of GEE or gingerol that resulted in ≥90% RAW 264.7 cells viability, was used for the next assay[9-14].

Induction of inflammation in RAW 264.7 cells and treatments

The inflammation condition for RAW 264.7 cells was triggered using lipopolysaccharide (LPS) (Sigma Aldrich, L2880) based on a modified method from Novilla *et al*[9] and Widowati *et al*[10]. Approximately, 5 x 10^3 of RAW 264.7 cells in DMEM supplemented with 10% FBS and 1% penstrep were seeded in a 6-well plate and incubated for 24 hours at 37 ^{o}C, 5% CO_2, humidified atmosphere. The prior medium was then washed and the cells were supplemented with 1.6 mL fresh medium and 200 µL of GEE or gingerol with a concentration based on the viability assay, incubated for 1-2 h. Subsequently, 200 µL of LPS (1 µg/mL) was added into each well except for the negative control well, incubated for 24 hours at 37 ^{o}C, 5% CO_2, and humidified atmosphere. The medium was then taken for TNF-α, IL-1β, IL-6, COX-2, and NO quantification, centrifuged at 2000 g for 10 minutes, and the supernatant was stored at -80 ^{o}C[9-14].

Measurement of TNF-α concentration and inhibitory activity in LPS-Induced RAW 264.7 cells

The measurement of TNF-α concentration was conducted based on the ELISA method, using Mouse TNF-α ELISA MAX™ Standard Kit (BioLegend, 430904) according to the manufacturer's protocol. The absorbance was measured at 450 nm. The inhibition activity was calculated based on the percentage of TNF-α concentration in each treatment towards the positive and negative control[9,11-14].

Measurement of IL-1β and IL-6 concentration and inhibitory activity in LPS-Induced RAW 264.7 cells

The measurement of IL-1β and IL-6 concentration was conducted based on ELISA method, using Mouse IL-1β ELISA MAX™ Standard Kit (BioLegend,

432604) and IL-6 ELISA MAX™ Standard Kit (BioLegend, 431301) respectively according to the manufacturer's instructions. The inhibition activity was calculated based on the percentage of IL-1β or IL-6 concentration in each of the treatments toward the positive and negative control[9-14].

Measurement of COX-2 concentration and inhibitory activity in LPS-Induced RAW 264.7 cells

The quantification of COX-2 used Mouse PTGS2/COX-2 ELISA kit protocol (Elabscience, E-EL-M0959). The absorbance was measured at 450 nm. The inhibition activity of treatments to COX-2 concentration was obtained from the percentage of COX-2 concentration in each treatment toward the positive and negative control[10,14].

Measurement of nitrite associated with NO and inhibitory activity in LPS-Induced RAW 264.7 cells

NO quantification using Abnova Kit (Abnova, KA 1342) was used to determine the concentration of the nitrite associated with NO production. The quantification was conducted as per manufacturer's protocol. The quantity of the nitrite was determined from the sodium nitrite standard curve. The LPS-stimulated cells without extract or gingerol were used as a positive control. The normal cell was used as a negative control[9-15].

Statistical analysis

The data were derived from three independent experiments, the value was presented as mean±standard deviation. Statistical analysis was performed using SPSS software (version 17.0). The significant differences were analyzed by analysis of variance (ANOVA) continued with Tukey HSD *post hoc* test, with $p < 0.05$ was considered as statistically significant.

Results

RAW 264.7 cells viability assay

The viability assay was conducted to determine the safe concentration of GEE and gingerol that is not toxic toward RAW 264.7 macrophage murine cell line. The assay was measured by MTS, based on the conversion of the yellow tetrazolium salt into a purple formazan product. The percentage of cell viability was calculated by comparing the cell viability value of treatments against the control. The concentration of treatments that resulted in the percentage of cell viability higher than 90% was considered as non-toxic against the cell used. In this study, the treatments that resulted in more than 90% of RAW 264.7 cells viable were GEE 10, 50 µg/mL and gingerol 10, 50 µg/mL (Table 1). Therefore, the respective concentration of treatments was used for the next assays.

TNF-α concentration and inhibitory activity

The TNF-α concentration and inhibitory activity of extract toward TNF-α in RAW 264.7 cell can be seen in Table 2. The percentage of inhibitory activity was calculated by comparing with the positive control. The treatments of GEE and gingerol were able to reduce the TNF-α concentration in the cell, but it depends on the concentration used. The GEE with a concentration of 50 µg/mL inhibited TNF-α concentration with inhibitory activity value of 53.09%, the highest among all treatments and comparable with the negative control which was normal RAW 264.7 cell without LPS induction. Gingerol 10 µM also showed a low concentration of TNF-α and inhibitory activity of TNF-α, with a value of 34.89 %.

Table 1 — Effect various concentrations of ginger ethanol extract and gingerol towards RAW264.7 cells viability

Treatments	Cell viability (%)
Control	100.00 ± 0.00^{b}
GEE 100 µg/mL	67.28 ± 13.19^{a}
GEE 50 µg/mL	103.11 ± 11.40^{b}
GEE 10 µg/mL	128.02 ± 12.02^{b}
Gingerol 100 µM	55.71 ± 7.67^{a}
Gingerol 50 µM	100.46 ± 9.34^{b}
Gingerol 10 µM	113.86 ± 16.90^{b}

Data were presented as mean±standard deviation, the experiment was conducted in triplicate (n=3). Different superscript letters (a,b) in the same column showed a significant difference among treatments at $p < 0.05$ (Tukey HSD post hoc test)

Table 2 — Effect various concentrations of ginger ethanol extract and gingerol towards TNF-α concentration in LPS-RAW264.7 cells

Treatments	TNF-α concentration (pg/mL)	TNF-α inhibitory activity over positive control (%)
Negative control	225.40 ± 10.54^{a}	-
Positive control	476.82 ± 43.27^{c}	-
GEE 50 µg/mL	223.70 ± 19.71^{a}	53.09 ± 4.13^{c}
GEE 10 µg/mL	470.25 ± 110.69^{c}	1.38 ± 23.22^{a}
Gingerol 50 µM	442.32 ± 53.03^{bc}	7.24 ± 11.12^{ab}
Gingerol 10 µM	310.46 ± 17.67^{ab}	34.89 ± 3.71^{bc}

Data were presented as mean±standard deviation, the experiment was conducted in triplicate (n=3). Different superscript letters in the same column (a, ab, bc, c) for TNF-α concentration and different superscript letters (a, ab, bc, c) for TNF-α inhibitory activity showed significant difference among treatments at $p < 0.05$ (Tukey HSD post hoc test)

IL-6 concentration and inhibitory activity

The IL-6 concentration was determined based on the ELISA method, and all treatments reduced the IL-6 concentration in LPS-induced RAW 264.7 cells (Table 3). The positive control (LPS-induced RAW 264.7) had the highest concentration of IL-6, indicating that LPS has succeeded in inducing the inflammation and increase the IL-6 concentration. GEE 50 µg/mL exhibited the highest IL-6 inhibitory activity, comparable to the normal cell (negative control). Gingerol also showed good IL-6 inhibitory activity, with over than 50% for both the concentration used (10 and 50 µM).

IL-1β concentration and inhibitory activity

The determination of the effect of GEE and gingerol toward IL-1β concentration in LPS-induced RAW 264.7 cells revealed that both treatments had moderate activity in reducing IL-1β levels. Based on data provided in Table 4, it can be seen that GEE 50 µg/mL had a greater ability to reduce IL-1β concentration than gingerol. The IL-1β inhibitory activity of GEE and gingerol showed that both are concentration-dependent. GEE 50 µg/mL had the highest activity among the treatments and did not differ significantly with negative control.

COX-2 concentration and inhibitory activity

The COX-2 concentration was reduced significantly in LPS-induced RAW 264.7 cells treated with either GEE or gingerol (Table 5). The GEE showed to have better COX-2 inhibitory activity compared to gingerol, with the highest inhibitory activity achieved by GEE 50 µg/mL. The negative control had the lowest COX-2 concentration, indicating that the normal cell of RAW 274.7 without inflammation induction produce a little amount of COX-2.

Nitrite associated with NO concentration and inhibitory activity

The nitrite concentration was associated with the NO concentration in the body, which can be used as an indication of inflammation. The positive control showed significantly higher NO concentration than the negative control, suggesting that induction of inflammation condition by LPS is successful (Table 6). In RAW 264.7 cells induced by LPS, it can be seen that the NO concentrations were relatively higher than the negative control, but the cells treated by either GEE or gingerol showed lower NO concentration than the positive control, indicating

Table 3 — Effect various concentrations of ginger ethanol extract and gingerol towards IL-6 concentration in LPS-RAW 264.7 cells

Treatments	IL-6 concentration (pg/mL)	IL-6 inhibitory activity over positive control (%)
Negative control	163.61±3.44^{a}	-
Positive control	512.67±2.77^{e}	-
GEE 50 µg/mL	164.32±3.09^{a}	70.16±0.56^{d}
GEE 10 µg/mL	300.11±3.81^{d}	45.50±0.69^{a}
Gingerol 50 µM	231.30±6.52^{b}	58.00±1.18^{c}
Gingerol 10 µM	250.38±4.27^{c}	54.53±0.77^{b}

Data were presented as mean±standard deviation, the experiment was conducted in triplicate (n=3). Different superscript letters in the same column (a, b, c, d, e) for IL-6 concentration and different superscript letters (a, b, c, d) for IL-6 inhibitory activity showed significant difference among treatments at p <0.05 (Tukey HSD post hoc test)

Table 4 — Effect various concentrations of ginger ethanol extract and gingerol towards IL-1β concentration in LPS-RAW264.7 cells

Treatments	IL-1β concentration (pg/mL)	IL-1β inhibitory activity over positive control (%)
Negative control	834.97±48.14^{a}	-
Positive control	1223.46±38.10^{c}	-
GEE 50 µg/mL	912.06±72.90^{a}	25.45±5.96^{c}
GEE 10 µg/mL	1207.67±0.77^{c}	1.29±0.06^{a}
Gingerol 50 µM	1038.54±26.90^{b}	15.11±2.20^{b}
Gingerol 10 µM	1136.40±46.55^{bc}	7.12±3.80^{ab}

Data were presented as mean±standard deviation, the experiment was conducted in triplicate (n=3). Different superscript letters in the same column (a, b, bc, c) for IL-1β concentration and different superscript letters (a, ab, b, c) for IL-1β inhibitory activity showed significant difference among treatments at p <0.05 (Tukey HSD post hoc test)

Table 5 — Effect various concentrations of ginger ethanol extract and gingerol towards COX-2 concentration in LPS-RAW264.7 cells

Treatments	COX-2 concentration (ng/mL)	COX-2 inhibitory activity over positive control (%)
Negative control	0.84±0.07^{a}	-
Positive control	2.41±0.07^{e}	-
GEE 50 µg/mL	1.65±0.07^{b}	31.67±2.76^{c}
GEE 10 µg/mL	1.83±0.04^{c}	23.93±1.46^{b}
Gingerol 50 µM	1.96±0.07^{cd}	18.67±2.91^{ab}
Gingerol 10 µM	2.12±0.04^{d}	12.03±1.66^{a}

Data were presented as mean±standard deviation, the experiment was conducted in triplicate (n=3). Different superscript letters in the same column (a, b, c, cd, d, e) for COX-2 concentration and different superscript letters (a, ab, b, c) for COX-2 inhibitory activity showed significant difference among treatments at p <0.05 (Tukey HSD post hoc test)

Table 6 — Effect various concentrations of ginger ethanol extract and gingerol towards COX-2 concentration in LPS-RAW264.7 cells

Treatments	NO concentration (pg/mL)	NO inhibitory activity over positive control (%)
Negative control	5.93±0.17[a]	-
Positive control	34.99±0.09[e]	-
GEE 50 µg/mL	24.23±0.40[b]	30.76±1.13[d]
GEE 10 µg/mL	25.75±1.17[b]	26.40±3.36[c]
Gingerol 50 µM	28.19±0.28[c]	19.44±0.80[b]
Gingerol 10 µM	30.29±0.73[d]	13.42±2.07[a]

Data were presented as mean±standard deviation, the experiment was conducted in triplicate (n=3). Different superscript letters in the same column (a, b, c, d, e) for COX-2 concentration and different superscript letters (a, b, c, d) for COX-2 inhibitory activity showed significant difference among treatments at p <0.05 (Tukey HSD post hoc test)

they have reduced NO level in the inflammatory condition. GEE 50 µg/mL showed highest NO inhibitory activity over positive control and gingerol 10 µM showed the lowest activity.

Discussion

In the present study, anti-inflammatory activities of GEE and gingerol was evaluated by measuring the production of TNF-α, IL-1β, IL-6, COX-2, and NO in LPS-induced RAW 264.7 murine macrophage cells.

LPS is a pro-inflammatory glycolipid component in the cell wall of Gram-negative bacteria, that has been reported to activate macrophage and boost the production of pro-inflammatory mediators including nitric oxide (NO), IL-1, IL-6, TNF-α, prostanoids, and leukotrienes[9-17]. These conditions proved in the present study revealed that the positive control which was the RAW 264.7 cells, induced by LPS had significantly higher TNF-α, IL-1β, IL-6, COX-2, and NO concentration compared to the negative control which was the RAW 264.7 cells without induction of LPS, indicating that LPS succeed in increasing the pro-inflammatory mediators.

To prevent an adverse effect of GEE and gingerol extract toward the RAW 264.7 cells, the viability assay was conducted and the result showed that GEE with the concentration of 10 and 50 µg/mL and gingerol 10 and 50 µM were safe for growth of the cells (Table 1). The GEE and gingerol dose-dependently inhibited the TNF-α, IL-1β, IL-6, COX-2, and NO production in LPS-induced RAW 264.7 macrophage cells (Table 2-6), suggesting that both possess anti-inflammatory properties. The GEE was observed to have higher inhibitory activity than gingerol, this might due to numerous phytochemicals and synergetic activity among compounds in the ginger besides gingerols such as shogaols and paradol that also have anti-inflammatory properties[2]. The TNF-α, IL-1β, IL-6, COX-2, and NO are pro-inflammatory cytokines and mediators that play roles in the inflammatory process, and inhibition of these molecules production are considered as anti-inflammatory activity[9-14].

The tumour necrosis factor α (TNF-α) is an inflammatory cytokine produced by macrophage in the inflammatory process[18], at the multicellular level it upregulates other pro-inflammatory cytokines (e.g. IL-6 and IL-1), induces angiogenesis, activates transcription factor NF-κB, and stimulates the production of NO[19-22]. IL-1β is a pro-inflammatory cytokine that could induce fever and secretion of IL-6 and IL-8 which are also pro-inflammatory cytokines[23]. IL-6 is a pleiotropic cytokine that modulates immune response and it activates neutrophils and NK cells[24,25]. Together with TNF-α and IL-1β, it could act as endogenous pyrogens which cause fever in inflammation by increasing the inflammatory response and stimulate the production of an acute phase reactant[22].

The pro-inflammatory cytokines, endotoxins, and mitogens could induce COX-2, an iso form of cyclooxygenase (COX) which is involved in the formation of prostaglandins (PG), a lipid mediators[26]. PGE_2 and PGI_2 play role in causing synovial inflammation by increasing local blood flow along with potentiating effects of bradykinin and IL-1[26]. Nitric oxide (NO) is a free radical molecule, it has a role in several physiological and pathophysiological processes including inflammation[27]. The NO is produced by inducible nitric oxide synthase (iNOS) from amino acid L-arginine[22], however, overproduction of NO in activated immune cells during inflammation could lead to major destructive effect in tissue injury[5].

The anti-inflammatory activities of ginger have been supported by other studies, Habib *et al*[2] reported that ginger extract has anti-cancer and anti-inflammatory properties by inactivating NK-κB through the pro-inflammatory TNF-α reduction in ethionine-induced hepatoma rats. Compounds from ginger namely 10-gingerol, 8-shogaol, and 10-shogaol are also able to inhibit COX-2 but not COX-1, showing its potential as selective inhibitors since inhibition of COX-1 may be correlated with gastrointestinal irritation[28]. Organic extracts of

gingerol are also capable of inhibiting LPS-induced PGE_2 production as reported by Lantz *et al*[29].

Conclusion

The research concludes that the ginger ethanolic extract and gingerol have anti-inflammatory properties by lowering the production of pro-inflammatory mediators such as TNF-α, IL-1β, IL-6, COX-2, and NO. The ginger ethanolic extract exhibited better anti-inflammatory than gingerol, showing its promising potential as therapeutic agents in the inflammatory related diseases treatment.

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Suppression of pro-inflammatory cytokines and mediators production by ginger (Zingiber officinale Roscoe) ethanolic extract and gingerol in lipopolysaccharide induced RAW 264.7 murine macrophage cells

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Suppression of pro-inflammatory cytokines and mediators production by ginger (*Zingiber officinale* Roscoe) ethanolic extract and gingerol in lipopolysaccharide-induced RAW 264.7 murine macrophage cells

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Chronic inflammation could lead to several life-threatening diseases such as cancer and cardiovascular diseases. Ginger (*Zingiber officinale* Roscoe) has been used for many years to treat various diseases and health problems, including inflammation. This study was conducted to assess ginger ethanolic extract (GEE) and its compound gingerol's potential as an anti-inflammatory agent by evaluating the concentration of pro-inflammatory cytokines and mediators such as TNF-α, IL-1β, IL-6, COX-2, and NO in LPS-induced RAW 264.7 cells. The safe concentration of GEE and gingerol for the RAW 264.7 cells were evaluated using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay. The quantification of TNF-α, IL-1β, IL-6, COX-2, was conducted based on the ELISA method, while the quantification of NO was conducted by the nitrate/nitrite colourimetric method. The results showed that GEE and gingerol were able to inhibit TNF-α, IL-1β, IL-6, COX-2, and NO production in LPS-induced RAW 264.7 cells. GEE has better anti-inflammatory activity than gingerol. GEE in the concentration of 50 μg/mL has the highest inhibition activity over positive control or inflammatory cells model. GEE exhibited good anti-inflammatory properties through reduction of pro-inflammatory mediators such as TNF-α, IL-1β, IL-6, COX-2 and NO. Thus, ginger ethanolic extract has a high potential in the treatment of inflammation-related diseases.

Keywords: Cytokines, Gingerol, Inflammation, Macrophage, *Zingiber officinale*.

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Introduction

Plants have long over the years been used as a popular mode of therapies for the treatment of numerous health problems[1]. One of the plants known to possess therapeutic properties is ginger (*Zingiber officinale* Roscoe), which also usually used as a spice in foods[2,3]. As a traditional herbal medicine, ginger is usually used to treat arthritis, rheumatism, sprains, muscular aches, pains, sore throats, cramps, constipation, indigestion, vomiting, hypertension, dementia, fever, infectious diseases, and helminthiasis[3]. The plant contains several active phenolic compounds such as gingerol, paradol, and shogoal proven to have antioxidant, anti-cancer, anti-angiogenesis, and anti-atherosclerotic activities[2]. The anti-inflammatory potential of ginger has also been reported by several studies[4].

Chronic inflammation has been popularly known to be linked with various diseases such as cardiovascular diseases, diabetes, arthritis, Alzheimer's disease, pulmonary diseases, cancer, and autoimmune diseases[2]. Inflammation is an innate response towards irritation and infection caused by pathogens, wounds, and chemicals, it is a complex process regulated by pro-inflammatory cytokines and mediators[5]. Macrophage, which is the cell line of chronic inflammation, is activated during inflammation by the exposure to interferon-γ (IFN-γ), pro-inflammatory cytokines, or bacterial lipopolysaccharides (LPS)[6-9]. The activated macrophage release several chemicals including reactive oxygen species (ROS), Nitric Oxide (NO), prostaglandin E (PGE), and cytokines (Interleukin-1β, Interleukin-6, and Tumor Necrosis Factor-α (IL-1β, IL-6, TNF-α) and cyclooxygenase-2 (COX-2)[7-9]. Overproduction of inflammatory mediators and cytokines in prolonged inflammation can cause cellular and tissue damages that lead to several diseases, therefore anti-inflammatory agents are important to prevent the side effect of prolonged inflammation and suppress the production of inflammatory marker molecules[8,9].

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In this study, the anti-inflammatory properties of ginger ethanolic extract (GEE) along with its compound gingerol assessed in LPS-induced RAW 264.7 murine macrophage cells as inflammation model by evaluating the TNF-α, IL-1β, IL-6, COX-2, and NO levels in the inflammatory cells model.

Materials and Methods

Plant extract preparation

The ginger (*Z. officinale* Roscoe) were collected from farmer plantation located in Bogor, West Java. The plant was identified by the staff of Herbarium, Department of Biology, School of Life Sciences and Technology, Bandung, West Java, Indonesia. The rhizome was chopped and dried using food dehydrator (40-45 °C) until stable water with level (±13%) was achieved. The dried rhizome of ginger was mashed and crushed into small pieces. Briefly, 200 mg of ginger powder was extracted by maceration technique with 70% ethanol solvent. Every 24 hours, the filtrate was collected until ethanol filtrate turned colourless. The filtrate was then evaporated using a rotary vacuum evaporator (Zhengzhou RE-201D) at a temperature 50 °C until extract was obtained in paste form. The paste product, which was ginger ethanolic extract (GEE) was stored at -20 °C for further use[9-14]. The gingerol (BP0092) were purchased from Chengdu Biopurify Phytochemical Ltd.

RAW 264.7 cells culture

The RAW 264.7 (ATCC®TIB-71™) murine macrophage cell line was obtained from Biomolecular and Biomedical Research Center, Aretha Medika Utama. The cells were grown in Dulbecco's Modified Eagle Medium (DMEM) (Biowest, L0104) supplemented with 20% Fetal Bovine Serum (FBS) (Biowest, S1810), 1% Penicillin-Streptomycin (Penstrep) (Biowest, L0022). The culture was incubated at 37 °C with 5% CO_2 in a humidified atmosphere until the cells were confluent (80-90%). The confluent cells then harvested using trypsin-EDTA (Biowest, X0930) and seeded on plates for the next assays[11,12].

Viability assay of RAW 264.7 cells toward GEE and gingerol

The determination of the number of viable cells was performed using MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay (Promega, G3582). Around 5 x 10^3 cells in 100 µL of medium (DMEM supplemented with 10% FBS and 1% Penstrep were plated on each well in 96-well plate, incubated for 24 hours at 37 °C, 5% CO_2, humidified atmosphere. The prior medium then washed and the cells supplemented with 90 µL of fresh medium and 10 µL of GEE (100, 50, 10 µg/mL) and gingerol (100, 50, 10 µM). After 24 hours, each well was added with 20 µL of MTS, incubated at 37 °C, 5% CO_2 for 3 hours. The absorbance was then measured at 490 nm with a spectrophotometer (Multiskan™ GO and Microplate Spectrophotometer, Thermo Scientific N12391). The untreated cells served as a control and the viability percentage was calculated from the viable cells from each treatment toward the control. The concentration of GEE or gingerol that resulted in ≥90% RAW 264.7 cells viability, was used for the next assay[9-14].

Induction of inflammation in RAW 264.7 cells and treatments

The inflammation condition for RAW 264.7 cells was triggered using lipopolysaccharide (LPS) (Sigma Aldrich, L2880) based on a modified method from Novilla *et al*[9] and Widowati *et al*[10]. Approximately, 5 x 10^3 of RAW 264.7 cells in DMEM supplemented with 10% FBS and 1% penstrep were seeded in a 6-well plate and incubated for 24 hours at 37 °C, 5% CO_2, humidified atmosphere. The prior medium was then washed and the cells were supplemented with 1.6 mL fresh medium and 200 µL of GEE or gingerol with a concentration based on the viability assay, incubated for 1-2 h. Subsequently, 200 µL of LPS (1 µg/mL) was added into each well except for the negative control well, incubated for 24 hours at 37 °C, 5% CO_2, and humidified atmosphere. The medium was then taken for TNF-α, IL-1β, IL-6, COX-2, and NO quantification, centrifuged at 2000 g for 10 minutes, and the supernatant was stored at -80 °C[9-14].

Measurement of TNF-α concentration and inhibitory activity in LPS-Induced RAW 264.7 cells

The measurement of TNF-α concentration was conducted based on the ELISA method, using Mouse TNF-α ELISA MAX™ Standard Kit (BioLegend, 430904) according to the manufacturer's protocol. The absorbance was measured at 450 nm. The inhibition activity was calculated based on the percentage of TNF-α concentration in each treatment towards the positive and negative control[9,11-14].

Measurement of IL-1β and IL-6 concentration and inhibitory activity in LPS-Induced RAW 264.7 cells

The measurement of IL-1β and IL-6 concentration was conducted based on ELISA method, using Mouse IL-1β ELISA MAX™ Standard Kit (BioLegend,

432604) and IL-6 ELISA MAX™ Standard Kit (BioLegend, 431301) respectively according to the manufacturer's instructions. The inhibition activity was calculated based on the percentage of IL-1β or IL-6 concentration in each of the treatments toward the positive and negative control[9-14].

Measurement of COX-2 concentration and inhibitory activity in LPS-Induced RAW 264.7 cells

The quantification of COX-2 used Mouse PTGS2/COX-2 ELISA kit protocol (Elabscience, E-EL-M0959). The absorbance was measured at 450 nm. The inhibition activity of treatments to COX-2 concentration was obtained from the percentage of COX-2 concentration in each treatment toward the positive and negative control[10,14].

Measurement of nitrite associated with NO and inhibitory activity in LPS-Induced RAW 264.7 cells

NO quantification using Abnova Kit (Abnova, KA 1342) was used to determine the concentration of the nitrite associated with NO production. The quantification was conducted as per manufacturer's protocol. The quantity of the nitrite was determined from the sodium nitrite standard curve. The LPS-stimulated cells without extract or gingerol were used as a positive control. The normal cell was used as a negative control[9-15].

Statistical analysis

The data were derived from three independent experiments, the value was presented as mean±standard deviation. Statistical analysis was performed using SPSS software (version 17.0). The significant differences were analyzed by analysis of variance (ANOVA) continued with Tukey HSD *post hoc* test, with p <0.05 was considered as statistically significant.

Results

RAW 264.7 cells viability assay

The viability assay was conducted to determine the safe concentration of GEE and gingerol that is not toxic toward RAW 264.7 macrophage murine cell line. The assay was measured by MTS, based on the conversion of the yellow tetrazolium salt into a purple formazan product. The percentage of cell viability was calculated by comparing the cell viability value of treatments against the control. The concentration of treatments that resulted in the percentage of cell viability higher than 90% was considered as non-toxic against the cell used. In this study, the treatments that resulted in more than 90% of RAW 264.7 cells viable were GEE 10, 50 μg/mL and gingerol 10, 50 μg/mL (Table 1). Therefore, the respective concentration of treatments was used for the next assays.

TNF-α concentration and inhibitory activity

The TNF-α concentration and inhibitory activity of extract toward TNF-α in RAW 264.7 cell can be seen in Table 2. The percentage of inhibitory activity was calculated by comparing with the positive control. The treatments of GEE and gingerol were able to reduce the TNF-α concentration in the cell, but it depends on the concentration used. The GEE with a concentration of 50 μg/mL inhibited TNF-α concentration with inhibitory activity value of 53.09%, the highest among all treatments and comparable with the negative control which was normal RAW 264.7 cell without LPS induction. Gingerol 10 μM also showed a low concentration of TNF-α and inhibitory activity of TNF-α, with a value of 34.89 %.

Table 1 — Effect various concentrations of ginger ethanol extract and gingerol towards RAW264.7 cells viability

Treatments	Cell viability (%)
Control	100.00±0.00^{b}
GEE 100 μg/mL	67.28±13.19^{a}
GEE 50 μg/mL	103.11±11.40^{b}
GEE 10 μg/mL	128.02±12.02^{b}
Gingerol 100 μM	55.71±7.67^{a}
Gingerol 50 μM	100.46±9.34^{b}
Gingerol 10 μM	113.86±16.90^{b}

Data were presented as mean±standard deviation, the experiment was conducted in triplicate (n=3). Different superscript letters (a,b) in the same column showed a significant difference among treatments at p <0.05 (Tukey HSD post hoc test)

Table 2 — Effect various concentrations of ginger ethanol extract and gingerol towards TNF-α concentration in LPS-RAW264.7 cells

Treatments	TNF-α concentration (pg/mL)	TNF-α inhibitory activity over positive control (%)
Negative control	225.40±10.54^{a}	-
Positive control	476.82±43.27^{c}	-
GEE 50 μg/mL	223.70±19.71^{a}	53.09±4.13^{c}
GEE 10 μg/mL	470.25±110.69^{c}	1.38±23.22^{a}
Gingerol 50 μM	442.32±53.03^{bc}	7.24±11.12^{ab}
Gingerol 10 μM	310.46±17.67^{ab}	34.89±3.71^{bc}

Data were presented as mean±standard deviation, the experiment was conducted in triplicate (n=3). Different superscript letters in the same column (a, ab, bc, c) for TNF-α concentration and different superscript letters (a, ab, bc, c) for TNF-α inhibitory activity showed significant difference among treatments at p <0.05 (Tukey HSD post hoc test)

IL-6 concentration and inhibitory activity

The IL-6 concentration was determined based on the ELISA method, and all treatments reduced the IL-6 concentration in LPS-induced RAW 264.7 cells (Table 3). The positive control (LPS-induced RAW 264.7) had the highest concentration of IL-6, indicating that LPS has succeeded in inducing the inflammation and increase the IL-6 concentration. GEE 50 μg/mL exhibited the highest IL-6 inhibitory activity, comparable to the normal cell (negative control). Gingerol also showed good IL-6 inhibitory activity, with over than 50% for both the concentration used (10 and 50 μM).

IL-1β concentration and inhibitory activity

The determination of the effect of GEE and gingerol toward IL-1β concentration in LPS-induced RAW 264.7 cells revealed that both treatments had moderate activity in reducing IL-1β levels. Based on data provided in Table 4, it can be seen that GEE 50 μg/mL had a greater ability to reduce IL-1β concentration than gingerol. The IL-1β inhibitory activity of GEE and gingerol showed that both are concentration-dependent. GEE 50 μg/mL had the highest activity among the treatments and did not differ significantly with negative control.

COX-2 concentration and inhibitory activity

The COX-2 concentration was reduced significantly in LPS-induced RAW 264.7 cells treated with either GEE or gingerol (Table 5). The GEE showed to have better COX-2 inhibitory activity compared to gingerol, with the highest inhibitory activity achieved by GEE 50 μg/mL. The negative control had the lowest COX-2 concentration, indicating that the normal cell of RAW 274.7 without inflammation induction produce a little amount of COX-2.

Nitrite associated with NO concentration and inhibitory activity

The nitrite concentration was associated with the NO concentration in the body, which can be used as an indication of inflammation. The positive control showed significantly higher NO concentration than the negative control, suggesting that induction of inflammation condition by LPS is successful (Table 6). In RAW 264.7 cells induced by LPS, it can be seen that the NO concentrations were relatively higher than the negative control, but the cells treated by either GEE or gingerol showed lower NO concentration than the positive control, indicating

Table 3 — Effect various concentrations of ginger ethanol extract and gingerol towards IL-6 concentration in LPS-RAW 264.7 cells

Treatments	IL-6 concentration (pg/mL)	IL-6 inhibitory activity over positive control (%)
Negative control	163.61±3.44^{a}	-
Positive control	512.67±2.77^{e}	-
GEE 50 μg/mL	164.32±3.09^{a}	70.16±0.56^{d}
GEE 10 μg/mL	300.11±3.81^{d}	45.50±0.69^{a}
Gingerol 50 μM	231.30±6.52^{b}	58.00±1.18^{c}
Gingerol 10 μM	250.38±4.27^{c}	54.53±0.77^{b}

Data were presented as mean±standard deviation, the experiment was conducted in triplicate (n=3). Different superscript letters in the same column (a, b, c, d, e) for IL-6 concentration and different superscript letters (a, b, c, d) for IL-6 inhibitory activity showed significant difference among treatments at p <0.05 (Tukey HSD post hoc test)

Table 4 — Effect various concentrations of ginger ethanol extract and gingerol towards IL-1β concentration in LPS-RAW264.7 cells

Treatments	IL-1β concentration (pg/mL)	IL-1β inhibitory activity over positive control (%)
Negative control	834.97±48.14^{a}	-
Positive control	1223.46±38.10^{c}	-
GEE 50 μg/mL	912.06±72.90^{a}	25.45±5.96^{c}
GEE 10 μg/mL	1207.67±0.77^{c}	1.29±0.06^{a}
Gingerol 50 μM	1038.54±26.90^{b}	15.11±2.20^{b}
Gingerol 10 μM	1136.40±46.55^{bc}	7.12±3.80^{ab}

Data were presented as mean±standard deviation, the experiment was conducted in triplicate (n=3). Different superscript letters in the same column (a, b, bc, c) for IL-1β concentration and different superscript letters (a, ab, b, c) for IL-1β inhibitory activity showed significant difference among treatments at p <0.05 (Tukey HSD post hoc test)

Table 5 — Effect various concentrations of ginger ethanol extract and gingerol towards COX-2 concentration in LPS-RAW264.7 cells

Treatments	COX-2 concentration (ng/mL)	COX-2 inhibitory activity over positive control (%)
Negative control	0.84±0.07^{a}	-
Positive control	2.41±0.07^{e}	-
GEE 50 μg/mL	1.65±0.07^{b}	31.67±2.76^{c}
GEE 10 μg/mL	1.83±0.04^{c}	23.93±1.46^{b}
Gingerol 50 μM	1.96±0.07^{cd}	18.67±2.91^{ab}
Gingerol 10 μM	2.12±0.04^{d}	12.03±1.66^{a}

Data were presented as mean±standard deviation, the experiment was conducted in triplicate (n=3). Different superscript letters in the same column (a, b, c, cd, d, e) for COX-2 concentration and different superscript letters (a, ab, b, c) for COX-2 inhibitory activity showed significant difference among treatments at p <0.05 (Tukey HSD post hoc test)

Table 6 — Effect various concentrations of ginger ethanol extract and gingerol towards COX-2 concentration in LPS-RAW264.7 cells

Treatments	NO concentration (pg/mL)	NO inhibitory activity over positive control (%)
Negative control	5.93±0.17[a]	-
Positive control	34.99±0.09[e]	-
GEE 50 μg/mL	24.23±0.40[b]	30.76±1.13[d]
GEE 10 μg/mL	25.75±1.17[b]	26.40±3.36[c]
Gingerol 50 μM	28.19±0.28[c]	19.44±0.80[b]
Gingerol 10 μM	30.29±0.73[d]	13.42±2.07[a]

Data were presented as mean±standard deviation, the experiment was conducted in triplicate (n=3). Different superscript letters in the same column (a, b, c, d, e) for COX-2 concentration and different superscript letters (a, b, c, d) for COX-2 inhibitory activity showed significant difference among treatments at p <0.05 (Tukey HSD post hoc test)

they have reduced NO level in the inflammatory condition. GEE 50 μg/mL showed highest NO inhibitory activity over positive control and gingerol 10 μM showed the lowest activity.

Discussion

In the present study, anti-inflammatory activities of GEE and gingerol was evaluated by measuring the production of TNF-α, IL-1β, IL-6, COX-2, and NO in LPS-induced RAW 264.7 murine macrophage cells.

LPS is a pro-inflammatory glycolipid component in the cell wall of Gram-negative bacteria, that has been reported to activate macrophage and boost the production of pro-inflammatory mediators including nitric oxide (NO), IL-1, IL-6, TNF-α, prostanoids, and leukotrienes[9-17]. These conditions proved in the present study revealed that the positive control which was the RAW 264.7 cells, induced by LPS had significantly higher TNF-α, IL-1β, IL-6, COX-2, and NO concentration compared to the negative control which was the RAW 264.7 cells without induction of LPS, indicating that LPS succeed in increasing the pro-inflammatory mediators.

To prevent an adverse effect of GEE and gingerol extract toward the RAW 264.7 cells, the viability assay was conducted and the result showed that GEE with the concentration of 10 and 50 μg/mL and gingerol 10 and 50 μM were safe for growth of the cells (Table 1). The GEE and gingerol dose-dependently inhibited the TNF-α, IL-1β, IL-6, COX-2, and NO production in LPS-induced RAW 264.7 macrophage cells (Table 2-6), suggesting that both possess anti-inflammatory properties. The GEE was observed to have higher inhibitory activity than gingerol, this might due to numerous phytochemicals and synergetic activity among compounds in the ginger besides gingerols such as shogaols and paradol that also have anti-inflammatory properties[2]. The TNF-α, IL-1β, IL-6, COX-2, and NO are pro-inflammatory cytokines and mediators that play roles in the inflammatory process, and inhibition of these molecules production are considered as anti-inflammatory activity[9-14].

The tumour necrosis factor α (TNF-α) is an inflammatory cytokine produced by macrophage in the inflammatory process[18], at the multicellular level it upregulates other pro-inflammatory cytokines (e.g. IL-6 and IL-1), induces angiogenesis, activates transcription factor NF-κB, and stimulates the production of NO[19-22]. IL-1β is a pro-inflammatory cytokine that could induce fever and secretion of IL-6 and IL-8 which are also pro-inflammatory cytokines[23]. IL-6 is a pleiotropic cytokine that modulates immune response and it activates neutrophils and NK cells[24,25]. Together with TNF-α and IL-1β, it could act as endogenous pyrogens which cause fever in inflammation by increasing the inflammatory response and stimulate the production of an acute phase reactant[22].

The pro-inflammatory cytokines, endotoxins, and mitogens could induce COX-2, an iso form of cyclooxygenase (COX) which is involved in the formation of prostaglandins (PG), a lipid mediators[26]. PGE_2 and PGI_2 play role in causing synovial inflammation by increasing local blood flow along with potentiating effects of bradykinin and IL-1[26]. Nitric oxide (NO) is a free radical molecule, it has a role in several physiological and pathophysiological processes including inflammation[27]. The NO is produced by inducible nitric oxide synthase (iNOS) from amino acid L-arginine[22], however, overproduction of NO in activated immune cells during inflammation could lead to major destructive effect in tissue injury[5].

The anti-inflammatory activities of ginger have been supported by other studies, Habib *et al*[2] reported that ginger extract has anti-cancer and anti-inflammatory properties by inactivating NK-κB through the pro-inflammatory TNF-α reduction in ethionine-induced hepatoma rats. Compounds from ginger namely 10-gingerol, 8-shogaol, and 10-shogaol are also able to inhibit COX-2 but not COX-1, showing its potential as selective inhibitors since inhibition of COX-1 may be correlated with gastrointestinal irritation[28]. Organic extracts of

gingerol are also capable of inhibiting LPS-induced PGE_2 production as reported by Lantz *et al*[29].

Conclusion

The research concludes that the ginger ethanolic extract and gingerol have anti-inflammatory properties by lowering the production of pro-inflammatory mediators such as TNF-α, IL-1β, IL-6, COX-2, and NO. The ginger ethanolic extract exhibited better anti-inflammatory than gingerol, showing its promising potential as therapeutic agents in the inflammatory related diseases treatment.

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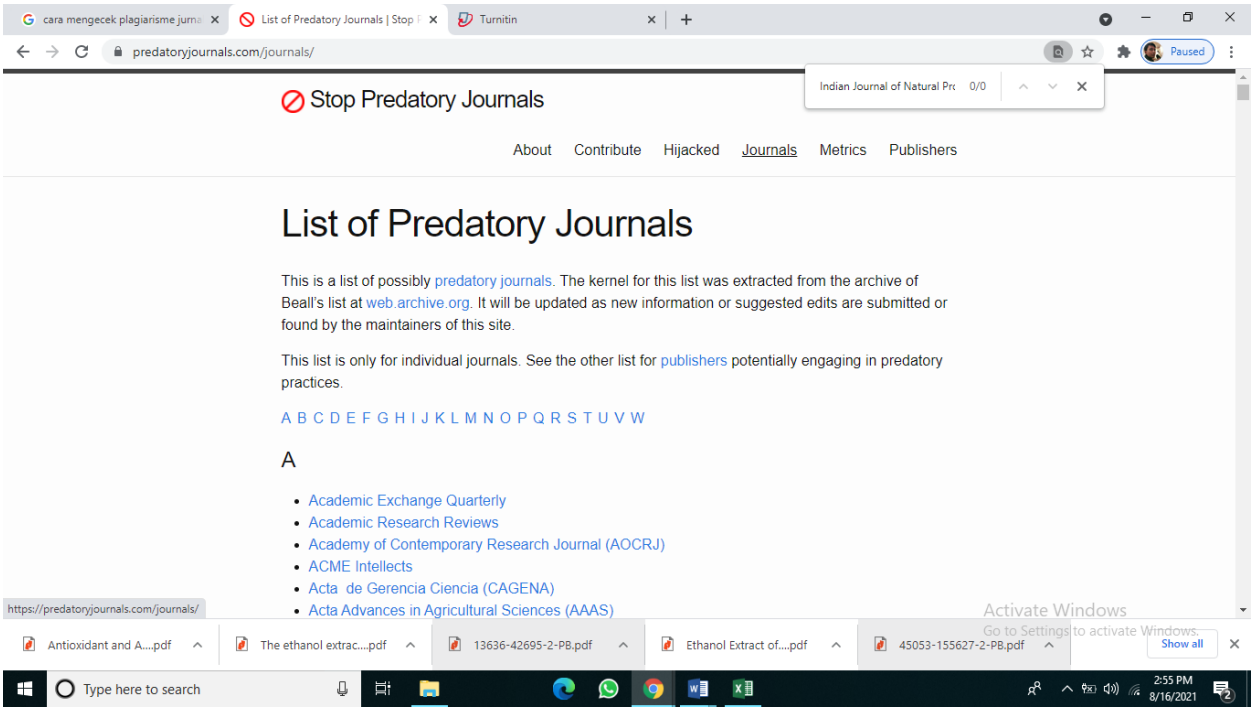
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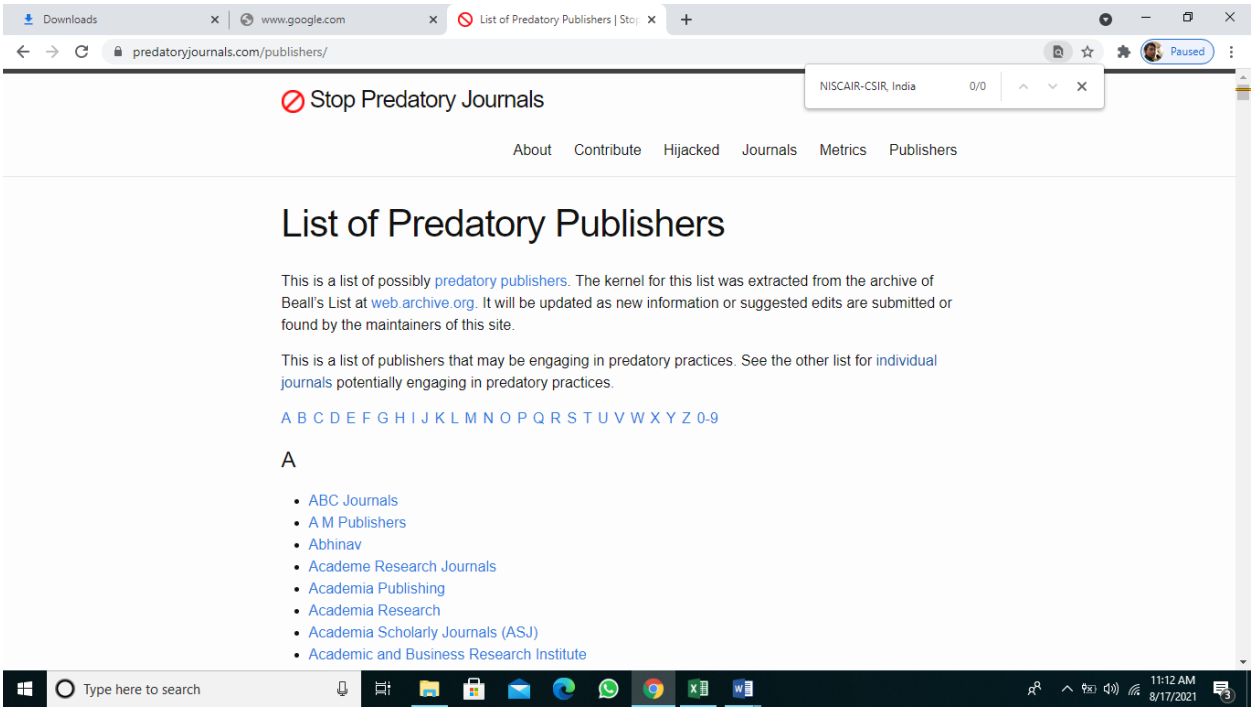
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