09.09.2020

SUMMARY OF REFEREES' COMMENTS AND EDITORIAL OBSERVATIONS ON IJNPR-2330-1266

Decision: Major Revision

Referees' and Editor's Comments

(MS with comments also attached)

1. Grammar should be thoroughly checked and corrected throughout the MS.
2. There is no inhibition in fact there is almost 3.5 times increase in IL-6 prodcution- specify this. The present data presentation in the table is confusing and does not provide clear picture about the activity
   Answer : The data presented in % about the comparison between positive control and treatment groups. The data about negative and positive control left blank.
3. The suppression of Pro-Inflammatory Cytokines and Mediators Production by Ginger in lipopoly saccharide induced / activated macrophage is already reported.
   Answer : If the journal from the title comment, there is a difference between this journal and that journal at the source of macrophage.
4. The authors are suggested to go through the following references and highlight the novelty of the current MS in the abstract as well as in conclusion, when compared to the already published research articles:

(i) *In vitro* anti-inflammatory activity of ginger(*Zingiber officinale* Rosc.) rhizome, callus and callus treated with some elicitors; Ammar Mohammed Ahmed Ali, Mawahib ElAmin Mohamed El-Nour, Owais Mohammad and Sakina Mohamed Yagi; Journal of Medicinal Plants Research; Vol. 13(10), pp. 227-235, 25 May, 2019.
Answer : The cell line used in this journal is different and the extraction method is different in the solvent used so the result should be different

(ii) Bioactive Compounds and Bioactivities of Ginger (Zingiber officinale Roscoe); Qian-Qian Mao, Xiao-Yu Xu, Shi-Yu Cao, Ren-You Gan , Harold Corke , Trust Beta and Hua-Bin Li 1,; foods; Foods 2019, 8, 185; doi:10.3390/foods8060185 www.mdpi
Answer : There is a difference in inhibiton of NO and PGE_2, meanwhile in the research done more than those.

(iii) Anti-neuroinflammatory Effects of 12-Dehydrogingerdione in LPS-Activated Microglia through Inhibiting Akt/IKK/NF-κB Pathway and Activating Nrf-2/HO-1 Pathway; Dong Zhao, Ming-Yao Gu, Jiu Liang Xu, Li Jun Zhang, Shi Yong Ryu and Hyun Ok Yang; Biomol Ther 27(1), 92-100 (2019)
Answer : The cell used was different and the compound used in treatment is different too even from ginger.

(iv) Anti-inflammatory effects of the Zingiber officinale roscoe constituent 12-dehydrogingerdione in lipopolysaccharide-stimulated Raw 264.7 cells; Young Ah Han, Chang Woo Song, Woo Suk Koh, Gyu Hwan Yon, Young Sup Kim, Shi Yong

Ryu, Hoon Jeong Kwon, Kyu Hong Lee; Phytotherapy Research; 2013 Aug; 27(8):1200-5; doi: 10.1002/ptr.4847. Epub 2012 Oct 2.
Answer : In this journal the compound used for treatment is the constituent of ginger different than the research was done.

(v) Anti-inflammatory effects of zingiber officinale roscoe involve suppression of nitric oxide and prostaglandin E2 production; Rizgar Maged, Nurul Nordin, Mohammed Sherwan Abdulla; Zanco J. Med. Sci., Vol. 17, No. (1), 2013, 349.
Answer : The extraction method was different and the pro-inflammatory detected in this journal is NO and PGE_2 only.

(vi) Anti-inflammatory action of ginger: A critical review in anemia of inflammation and its future aspects; Subodh Kumar, Kiran Saxena, Uday N. Singh, Ravi Saxena; International Journal of Herbal Medicine; 2013; 1 (4): 16-20.
Answer : This review only review about the role of ginger and active compound inhibit of TNF-α in vivo not in cell culture.

5. Add recent references from 2016 to 2020 in the MS.
   Answer : There was no need to add the recent references if there was none recent references that has connection with this research
6. Abstract:…….. GEE has better anti-inflammatory activity than gingerol, with GEE in concentration of 50 µg/ml has the highest inhibition activity over positive control. Ginger (*Zingiber officinale*) ethanolic extract exhibited good anti-inflammatory properties through reduction of pro-inflammatory mediators such as TNF-α, IL-1β, IL-6, COX-2 and NO…...
   Why GEE showed higher activity than gingerol. Whether GEE contained a higher active compound than gingerol/ is it synergistic effect.
   Answer : This question already answered in Discussion paragraph 3 that ginger has numerous phytochemicals than gingerol that have anti-inflammatory properties.
7. Provide HPLC profile of GEE and identify the active compounds present in GEE.
   Answer : We didn't conduct HPLC for GEE but the active compound we found from the journal. You can see the citation at page 5 highlighted.
8. Materials and methods: The dried rhizome of ginger…..
   Provide brief details – how zinger is dried? What is the moisture content of dry ginger?
   Answer: The rhizome were dried using food dehydrator (40-45^{o}C) until achieve stable water with level (±13%).
9. Whether the anti-inflammatory activity of GEE / gingerol was compared with any of the standard drugs available in the market? If yes, name it. If not, why it is not compared.
   Answer : We didn't use the standard drugs, because we wanted to compare the ginger extract efficacy with pure compound that is gingerol
10. Expand: FBS like areviations.
    Answer: There has been changed in manuscript
11. Please follow the IJNPR format.
    Answer: We has been followed the guideline
12. Please ensure that the author name, affiliation and address are complete (with pin code) and are correct.
    Answer: We apologize but the title page in separate file, not for review.
13. Keywords must be alphabetically arranged.
    Answer : There has been changed in manuscript

Editorial Requirement

1. Point-wise reply to comments should be given along with revised manuscript (highlighting the changes made).
2. Paper is to be modified as per the reviewer's suggestions.
3. Complete the references and check whether by mistake same ref has been numbered twice. Volume and pages should be there. **Ref. are to be given in Numerical System otherwise it would not be processed further.**
4. Tables and fig are to be numbered properly.
5. Provide complete legends and headings of tables and fig/plates. **Fig and plates to be sent in jpg file.**
6. Provide Running title and Keywords properly.
7. Place all tables and Fig/Plate at the end i.e. after references, not in between the text matter.
8. Avoid many tables; try to merge information in text.
9. Reply point wise all queries of Reviewers.
10. Submit revised manuscript by Email and OJS.
11. REVISED PAPER SHOULD BE STRICTLY IN THE FORMAT OF IJNPR otherwise it would be delayed in publication or Rejected.
12. The paper may be accepted after the suggestions/corrections pointed out in the text and in this report are incorporated.

With best regards
Editor, IJNPR

Suppression of Pro-Inflammatory Cytokines and Mediators Production by Ginger (*Zingiber officinale*) ~~Ethanolic~~ Ethanolic Extract and Gingerol in Lipopolysaccharide-Induced RAW264.7 ~~Murine Macrophage~~ Murine Macrophage Cells

ANTI-INFLAMMATORY ACTIVITIY OF GINGER AND GINGEROL

Abstract

Chronic inflammation could lead to several life-threatening diseases such as cancer and cardiovascular diseases. Ginger (*Zingiber* officinale) 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 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, and NO was conducted based on ELISA method. 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, with GEE in concentration of 50 µg/ml has the highest inhibition activity over positive control. Ginger (*Zingiber officinale*) ethanolic extract exhibited good anti-inflammatory properties through reduction of pro-inflammatory mediators such as TNF-α, IL-1β, IL-6, COX-2 and NO. Thus, it has high potential in the treatment of inflammation ~~ory~~-related diseases.

Keywords: Cytokines, gingerol, inflammation, macrophage, *Zingiber officinale*~~, gingerol, cytokines, macrophage~~

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Published online 2008 Jan 3. doi: 10.1186/1472-6882-8-1
PMCID: PMC2234390
Ginger extract inhibits LPS induced macrophage activation and function
Sudipta Tripathi,[1] David Bruch,[1] and Dilip S Kittur
What is new in this paper? Perhaps evaluation of gingerol's effect.

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Introduction

Plants have long over the years been used as popular mode of therapies for treatment of numerous health problems[1]. One of the plants that has been known to possess therapeutic properties is ginger (*Zingiber officinale* Roscoe), which also usually used as spice in foods[2,3]. As traditional herbal medicine~~s~~, 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 that proven to have antioxidant, anti-cancer, anti-angiogenesis, and anti-artherosclerotic activities[2]. The anti-inflammatory potentials 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 ~~immune~~ 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 primary cell of chronic inflammation, is activated during inflammation by the exposure to interferon-γ (IFN-γ), pro-inflammatory cytokines, or bacterial lipopolysaccharides (LPS)[6]. The activated macrophage release~~d~~ several chemicals including reactive oxygen species (ROS), Nitric Oxide (NO), prostaglandin, and cytokines (Interleukin-1β, Interleukin-6, and Tumor Necrosis Factor-α (IL-1β, IL-6, TNF-α))[7]. 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 production of these molecules[8,9].

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In this study, we assessed the anti-inflammatory properties of ginger ethanolic extract along with its compound gingerol 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 cells.

Materials and Methods

Plant Extract Preparation

The ginger (Zingiber officinale Roscoe) were collected from farmer plantation located in Bogor, West Java. The plant were identified by the staff of Herbarium, Department of Biology, School of Life Sciences and Technology, Bandung, West Java, Indonesia. The rhizome were chopped and dried using food dehydrator (40-45ºC) until achieve stable water with level (±13%). The dried rhizome of ginger (*Zingiber officinale*) was mashed and crushed into small pieces~~, then extracted using distillated ethanol 70%. The extraction was performed by simple maceration method, filtrate was filtered and collected every 24 h until it became colorless. The collected filtrate was evaporated at 40 ºC using an evaporator until the pasta form product was obtained.~~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 colorless. The filtrate was then evaporated using a rotary vacuum evaporator at a temperature 50ºC until extract obtained in paste form. The pasta product, which was ginger ethanolic extract (GEE), stored at -20 ºC for further use[10]. 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 ~~DMEM (~~Dulbecco's Modified Eagle Medium (DMEM)~~)~~ (Biowest) supplemented with 20% Fetal Bovine Serum (FBS) (Biowest), 1% penicillin-streptomycin (Biowest). 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), and seeded on plates for the next assays[11,12].

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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, Madison, WI, USA). Around 5 x 10^3 cells in 100 µl of medium (DMEM supplemented with 10 % Fetal Bovine Serum (FBS) and 1% penicillin-streptomycin) were plated on each well in 96-well plate, incubated for 24 h at 37 ^oC, 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) or gingerol (100, 50, 10 µM). After 24 h, each well was added with 20 µl of MTS, incubated at 37 ^oC, 5% CO_2 for 3 h. The absorbance then measured at 490 nm with spectrophotometer (Multiskan GO and µdrop Plate Thermo Scientific, Model N12391). The untreated cells were served as 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% or more of RAW 264.7 cells viable was used for the next assay[10-13].

Induction of Inflammation in RAW 264.7 Cells and Treatments

The inflammation condition for RAW 264.7 cells was triggered using lipopolysaccharide (LPS) (Sigma) based on modified method from Yoon *et al*[9] and Khan *et al*[14]. Approximately, 5 x 10^3 of RAW 264.7 cells in DMEM supplemented with 10% Fetal Bovine Serum (FBS) and 1% penicillin-streptomycin were seeded in a 6-well plate, incubated for 24 h at 37 ^oC, 5% CO_2, humidified atmosphere. The prior medium then washed and the cells were supplemented with 1.6 ml fresh medium and 200 µl of GEE or gingerol with concentration based on the viability assay, incubated for 1-2 h. Subsequently, 200 µl of LPS (1µg/ml) was added into each well except for the negative control well, incubated for 24 h at 37 ^oC, 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 min. The supernatant was stored at -79 ^oC[11].

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 ELISA method, using Mouse TNF-α ELISA MAX™ Standard Kit (BioLegend) according to the manufacturer's protocol. The absorbance

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was measured at 450 nm. The inhibition activity was calculated based on the percentage of TNF-α concentration in each ~~treatments~~ treatment towards the positive and negative control[11].

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) and IL-6 ELISA MAX™ Standard Kit (BioLegend) 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 treatments toward the positive and negative control[9,11].

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). The absorbance was measured at 450 nm. The inhibition activity of treatments to COX-2 concentration was obtained from percentage of COX-2 concentration in each ~~treatments~~ treatment toward the positive and negative control[15].

Measurement of Nitrite Associated with NO and Inhibitory Activity in LPS-Induced RAW 264.7 Cells

Abnova Kit (No cat. KA 1342) was used to determine the concentration of nitrite associated with NO production. The quantification was conducted as per manufacturer's protocol. The quantity of nitrite was determined from sodium nitrite standard curve. The LPS-stimulated cells without extract or gingerol was used as positive control. The normal cell was used as negative control[11,15].

Statistical Analysis

The data was derived from three independent experiments, value presented as mean ± standard deviation. Statistical analysis was performed using SPPS 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.

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Results

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RAW 264.7 cells viability assay

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The viability assay was conducted to determine the safe concentration of GEE or gingerol that is not toxic toward RAW 264.7 macrophage murine cell line. The assay was measured by MTS, based on the conversion of 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 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

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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 50 with concentration of 50 µg/ml showed to be able to inhibit 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 low concentration of TNF-α and inhibitory activity of TNF-α, with value of 34.89 %.

IL-6 concentration and inhibitory activity

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

IL-1β concentration and inhibitory activity

The determination of 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 clearly seen that GEE 50 µg/ml had greater ~~ablity~~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, ~~seen to be~~ and did not differ significantly with the 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 highest inhibitory activity achieved by GEE 50 µg/ml. The negative control had the least COX-2 concentration, indicating that the normal cell of RAW 274.7 without inflammation induction produce 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 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 ~~was succeed~~ (table 6). In ~~all~~ 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 ~~it were succeed~~ they have ~~in~~ ~~inhibited~~ reduced NO level in inflammatory ~~ion~~ condition. GEE 50 µg/ml showed highest NO inhibitory activity over positive control, and gingerol 10 µM showed the lowest activity.

Discussion

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

Lipopolysaccharide (LPS) is a pro-inflammatory glycolipid component in the cell wall of Gram negative bacteria, that has been reported could activate macrophage and boost the production of pro-inflammatory mediators including nitric oxide (NO), IL-1, IL-6, TNF-α, prostanoids, and leukotrienes[16,17]. These condition also proved in this 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.

In order to prevent adverse effect of GEE and gingerol extract toward the RAW 264.7 cells, the viability assay was conducted, the result showed that GEE with concentration of 10 and 50 µg/ml and gingerol 10 and 50 µM were safe for growth of the cells (table 1). The ginger ethanolic extract (GEE) and gingerol was found to dose-dependently inhibit 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 revealed had higher inhibitory activity than gingerol, this might due to numerous phytochemicals 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].

The tumor 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 production of NO[19-22].

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IL-1β is a pro-inflammatory cytokines that could induce~~s~~ fever and secretion of IL-6 and IL-8 which are also pro-inflammatory cytokines[23]. IL-6 is 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 ~~ion~~ response and stimulate production of acute phase reactant[22].

The pro-inflammatory cytokines, endotoxins, and mitogens could induce COX-2, an isoform of cyclooxygenase (COX) which is involved in the formation of ~~formed~~ 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~~s~~, it has 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 ~~forces~~ effect in tissue injury[5].

The anti-inflammatory activities of ginger also 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]. In addition, organic extracts of gingerol were capable ~~to~~ of~~–~~ inhibiting LPS-induced PGE_2 production as reported by Lantz *et al*[29].

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~~Conclusion~~

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The conclusion of this research is ~~T~~the ginger ethanolic extract and gingerol have anti-inflammatory properties by lowering production of pro-inflammatory mediators such as TNF-α, IL-1β, IL-6, COX-2, and

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NO. The ginger ethanolic extract exhibited better anti-inflammatory than gingerol, showing its promising potential as therapeutic agents in inflammatory related diseases treatment.

Acknowledgments

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~~**Conflict Of Interest**~~

~~The authors declare that they have no conflict of interest.~~

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Tables

Table 1--Mean and Tukey HSD post hoc test of RAW 264.7 cell viability towards GEE and gingerol measured in triplicate

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 (n=3). Different superscript letters (a,b) in the same column showed significant difference between different samples and concentrations at $P < 0.05$ (Tukey HSD post hoc test)

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Table 2--Mean and Tukey HSD post hoc test of TNF-α in RAW 264.7 cell treated with various concentration of GEE and gingerol measured in triplicate

Treatments	TNF-α concentration (pg/mL)	TNF-α inhibitory activity over positive control (%)
Negative control	225.40±10.54^{a}	~~52.73±2.21^{c}~~ -
Positive control	476.82±43.27^{c}	~~0.00±9.07^{a}~~ -
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 (n=3). Different superscript letters (a, b, c) in the same column showed significant difference between different samples and concentrations at $P < 0.05$ (Tukey HSD post hoc test)

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Commented [d10]: See the comment above provide what is meant by a, b, c- the significance with respect to which group has to be clearly mentioned.
There is no inhibition on the contrary there is more than two fold increase- this has to be specified

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Table 3--Mean and Tukey HSD post hoc test of IL-6 in RAW 264.7 cell treated with various concentration of GEE and gingerol measured in triplicate

Treatments	IL-6 concentration (pg/mL)	IL-6 inhibitory activity over positive control (%)
Negative control	163.61 ± 3.44[a]	~~70.29 ± 0.62[e]~~
Positive control	512.67 ± 2.77[e]	~~6.90 ± 0.50[a]~~
GEE 50 µg/mL	164.32 ± 3.09[a]	70.16 ± 0.56[e]
GEE 10 µg/mL	300.11 ± 3.81[d]	45.50 ± 0.69[b]
Gingerol 50 µM	231.30 ± 6.52[b]	58.00 ± 1.18[d]
Gingerol 10 µM	250.38 ± 4.27[c]	54.53 ± 0.77[c]

Data were presented as mean ± standard deviation (n=3). Different superscript letters (a, b, c, d) in the same column showed significant difference between different samples and concentrations at $P < 0.05$ (Tukey HSD post hoc test)

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Commented [d12]: There is no inhibition in fact there is almost 3.5 times increase in IL-6 prodcution- specify this. The present data presenation in the table is confusing and does not provide clear picture about the activity

Commented [AR13R12]: The inhibition measured by compared the positive control data with the treatment group in % The positive and negative control shouldn't be compared with positive control, so we left it blank

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Table 4--Mean and Tukey HSD post hoc test of IL-1β in RAW 264.7 cell treated with various concentration of GEE and gingerol measured in triplicate

Treatments	IL-1β concentration (pg/mL)	IL-1β inhibitory activity over positive control (%)
Negative control	834.97±48.14[a]	~~31.75±3.94[c]~~ -
Positive control	1223.46±38.10[c]	~~0.00±3.11[a]~~ -
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 (n=3). Different superscript letters (a, b, c) in the same column showed significant difference between different samples and concentrations at P < 0.05 (Tukey HSD post hoc test)

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Commented [d14]: See the comments related to Table 1-3 and revise the table accordingly
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Table 5--Mean and Tukey HSD post hoc test of COX-2 in RAW 264.7 cell treated with various concentration of GEE and gingerol measured in triplicate

Treatments	COX-2 concentration (ng/mL)	COX-2 inhibitory activity over positive control (%)
Negative control	0.84 ± 0.07^{a}	~~65.01 ± 2.70^{e}~~ -
Positive control	2.41 ± 0.07^{e}	~~0.14 ± 2.76^{a}~~ -
GEE 50 µg/mL	1.65 ± 0.07^{b}	31.67 ± 2.76^{d}
GEE 10 µg/mL	1.83 ± 0.04^{c}	23.93 ± 1.46^{c}
Gingerol 50 µM	1.96 ± 0.07^{cd}	18.67 ± 2.91^{bc}
Gingerol 10 µM	2.12 ± 0.04^{d}	12.03 ± 1.66^{b}

Data were presented as mean ± standard deviation (n=3). Different superscript letters (a, b, c, d, e) in the same column showed significant difference between different samples and concentrations at P < 0.05 (Tukey HSD post hoc test)

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Table 6--Mean and Tukey HSD post hoc test of NO in RAW 264.7 cell treated with various concentration of GEE and gingerol measured in triplicate

Treatments	NO concentration (pg/mL)	NO inhibitory activity over positive control (%)
Negative control	5.93±0.17[a]	~~83.04±0.49[e]~~ -
Positive control	34.99±0.09[e]	~~0.00±0.24[a]~~
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[d]
Gingerol 50 µM	28.19±0.28[c]	19.44±0.80[c]
Gingerol 10 µM	30.29±0.73[d]	13.42±2.07[b]

Data were presented as mean ± standard deviation (n=3). Different superscript letters (a, b, c, d, e)in the same column showed significant difference between different samples and concentrations at $P < 0.05$ (Tukey HSD post hoc test)

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