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Manuscript Title: **Anti-inflammatory Activities of Coumarin and Indonesian Cassia (Cinnamomum burmannii (C. Nees & T. Ness)) Extract in RAW264.7 Murine Macrophage Cell Line**

Authors: Ni Made Sandhiutami, Moordiani Moordiani, Dian Ratih Laksmitawati, Nurul Fauziah, Maesaroh Maesaroh, Wahyu Widowati

Dear Dr **Dr. Wahyu Widowati**

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I have been asked to review the article titled as follows; “Anti-inflammatory activities of coumarin and Indonesian Casia (Cinnamomum burmannii 9C.Nees & T.Nees)) extract in RAW264.7 murine macrophage cell line”. In overall, regardless of determination of cytokine levels by ELISA and some minor modifications , there is no novel knowledge regarding the present study. There are two previously published studies (1: Pharmacological mechanism underlying anti-inflammatory properties of two structurally divergent coumarins through the inhibition of pro-inflammatory enzymes and cytokines, in 2015 and 2: Anti-inflammatory activities of cinnamomum cassia constituents in vitro and in vivo, in 2012) with comparable results and conclusion with the present research.

However, if the editorial board and editor in chief of IJBMS, are interested about the publication of this manuscript, my comments are listed below for revision.

- The title of the manuscript should be changed as follows: “In vitro assessment of anti-inflammatory activities of coumarin and Indonesian Casia extract in RAW264.7 murine macrophage cell line”.

  Thank you for the recommendation. We have been revised the title of manuscript.

- The author should not present reference for the manuscript aim. What does it mean the reference 15 for the aim?

  We have been removed the reference (15) on manuscript aim.

- What is the full name for the abbreviation of MTS in page 3? The full name should be written for the first time.

  MTS stands for (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium). We have been added the full name in manuscript.

- In methods, please write the concentration of MTS instead of the volume (20 µl).

Protocol sheet oof MTS Cell Proliferation Assay only provide information as following: Reagent contains a novel tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS(a)] and an electron coupling reagent (phenazine ethosulfate; PES). The concentrations of tetrazolium and electron transfer reagents have been optimized for general use with a wide variety of cell lines cultured in 96-well plates containing 100 µl of medium. If different volumes of culture medium are used, adjust the volume to maintain a ratio of 20µl Reagent per 100µl culture medium.

- There is not clear what is the purpose of the author to bring reference of 15 and 16 and also 8 and 9, 22 and 19, in the methodology. Please explain it clearly.

  We have re-arranged the order of references accordingly. Reference (15) and (16) were cited for extraction method, (17) and (18) for cell culture, (17-19) for viability cell, (18) and (19) for pro-inflammatory activation, PGE_2 and NO assay, (18) and (20) for IL-6 assay, (8) and (18) in IL-1β, and (18) and (21) for TNF-α assay

- The methodology for measurement of cytokines (TNF-a and IL-1beta) by ELISA Kit from eBiolegend company should be written briefly using the sentence as follows; IL-1 beta and TNF-alpha levels in supernatant were determined by ELISA technique according to the manufacture's instruction manual (eBiolegend,….).

  We have stated that measurement of cytokines (TNF-a and IL-1beta) was performed according to protocol from eBiolegend company in method.

- Explanation about the abbreviations of a, b, c, d, e and f letters inside of table 1,2,3 and etc should write down In the footnote of that tables. Using these different letters without explanations, are too confusing for readers. They might use repeated measurement ANOVA, if they aim to compare more than two variables simultaneously.

  Tables has been replaced with Graphs with clear caption below.

- I think the number of tables are too much. It would be much better to show data in graphs.

  Tables has been replaced with Graphs. Thank you for the recommendation.

- The manuscript does not enough power to publish as an original article. It might be revised and submitted again as a short communication.

We believe that our research provided novel knowledgement and sufficient data, which is suitable as original article. Compared to previous study in 2005 "Pharmacological mechanism underlying anti-inflammatory properties of two structurally divergent coumarins through the inhibition of pro-inflammatory enzymes and cytokines", our study observed different kind of coumarin. We also observed various inflammatory markers compared to previous study. Moreover, our present study showed stronger anti-inflammatory activities compared to previous

study of *Cinnamomum cassia* in vitro and in vivo (2012). We used Indonesia cassia which is abundantly found in our country.

Dear Dr. Bibi Sedigheh Fazly Bazza, PhD.,

I am sending the review on the work of IJBMS-1603-4323.
The authors studied the anti-inflammatory effects of ICE and coumarin in RAW264.7 cell line. It is an interesting topic and I have some comments and questions for the authors:

Material and methods
Extract preparation (page 3)
The authors collected two filtrates after maceration of bark. From the text, it is not clear, if each filtrate was examined separately or if they were joined together. Why the bark was macerated twice? Can there exist differences in the concentration of studied substances?

Response: Maceration was repeated until the whole crude extract became colorless. The filtrates obtained from maceration, were joined together. We have stated in sentence ".....the filtrates were collected and the residues were immersed again in 70% distilated ethanol for 24 h. These treatments were repeated until the filtrate became colorless. *The collected filtrates* were evaporated...." There might be difference in concentration of substance in each filtrate, but we did not measure. We only aimed to collect extract with compounds contained in it as much as possible.

There is no information concerning coumarin preparation, if it was isolated from the extracts (using which method?) or if it was a commercial preparation. If it is possible, it should also be written, which type of coumarin the authors used.

Response: The coumarin was purchased from Chengdu [91-64-5]

Cell culture (page 3)

Abbreviation FBS should be explained

Response: The abbreviation FBS has been explained.

RAW264 cell viability assay (page 3)
At the beginning of this part, I recommend to shortly explain the principle of used method, as it was described in the part Results on page 7 and elsewhere in further papers as well.

Response: The principle method of cell viability has been added.

The authors wrote that they added 10 µl of extract (page 3, last line). However, they did not mention, in which solution the evaporated extract was solved (distilled water or DMSO?).

Response: Extract was solved in DMSO for treatment.

Page 3, last line: the authors wrote that they added 10 µl of extract (ICE) and coumarin. It is not clear, if the mentioned 10 µl contained 10, 50 and 100 µg of both studied substances (ICE and coumarin). Because, in Tables, there is written only used concentration 10ug/ml, 50 µg/ml and 100 µg/ml.

Response: The concentration of ICE and coumarin (10, 50 and 100 µg/ml; µM), has been mentioned in method

Pro-inflammatory activation (page 3)

The authors mentioned a slight modification of the method. It should be emphasized what kind of modification it was.

Response: Method used in the measurement was taken from Rusmana et al (2015) and Dewi et al (2015), which is a modified method from Yoon and Khan. Thus, we have stated and cited both Rusmana and Dewi in the method.

Which serotype of LPS was used?

Response: LPS used in this study was LPS from *E-coli* [Sigma, L2880]. The information has been added in Method.

Before adding of ICE and coumarin to medium, it should be completed, in which solution ICE and coumarin were dissolved after evaporation.

Response: ICE and coumarin were dissolved in DMSO.

Page 3, line 8: „The cells were plated in 6-well plate, ...“ – there should be written, I suppose, 96-well plate.

Response: There was no mistakes in the sentence. We used 6-well plate for pro-inflammatory activities. We used 96-well plate in cytotoxic assay.

I suggest explaining shortly the calculation of values concerning positive and negative activity.

Response: calculation of values regarding inhibitory activity over positive and negative control, has been briefly described in footnotes below figure.

Results
The numbering of Tables 5 and 6, concerning IL-2 and TNF-α, should be corrected.

Response: Tables has been replaced with Figures.

Table 1
As I already mentioned, it is not known in which solution ICE and coumarin were dissolved. Therefore, in Table 1, if Control value corresponds to the sample dissolved in DMSO, then Table should contain absorbance value with and without DMSO.

Response: Control used was cells+medium without extracts and coumarin.

All Tables: there is missing explanation of letters a-f, what is the significance of a-f against what?

Response: For better understanding, authors decided to replace Tables with Figures..

Tables 3-7: I recommend completing the title Positive control as „Positive control (LPS)“ to better understand differences between Negative and Positive controls in each Table.

Response: Positive control has been described as LPS-stimulated RAW 264.6 cells in method and result.
.
Tables 3-7: The authors defined the inhibition activity of positive and/or negative controls as 0 %. I recommend expression of controls as 100% and then to calculate the activity levels. The authors should explain why the values of inhibition activity of positive control are negative in the Tables.

Response: Inhibitory activity over positive control was measured based on comparison between treatment and positive control (positive control-treatment/positive control*100%), whilst inhibitory activity over negative control was measured based on comparison between treatment and negative control (negative control-treatment/negative control*100%). Since inhibition by treatments were comparable to negative control, negative values were possibly obtained as our result. However, we have provided data in Graphs to be easily interpreted that displays inhibitory activity of treatment and its comparison to positive control.

Discussion

The authors studied anti-inflammatory effects of ICE and coumarin. As in some cases the authors observed stronger anti-inflammatory effects of ICE compared to coumarin, they should explain the possible reasons. The anti-inflammatory effects of coumarin on levels of inflammatory mediators have been already studied in different works using RAW264 cell line as well. Therefore, it would be interesting to mention, if there could exist some differences in anti-inflammatory activities among coumarins isolated from various plants.

Response: Thank you for the recommendation. We have described possible reason regarding higher activity of ICE compared to coumarin, which is associated with constituents in extract. We have also provided the results of previous studies that can be comparison of anti-inflammatory activities of coumarin in the present study.

References

I recommend unifying the way of writing the names of authors and to control grammar.

Response: References has been written accordingly.

ABSTRACT

Background : Inflammation is an immune response toward injuries. Although inflammation is healing response, but in the some condition it will lead to chronic disease such as rheumatoid arthritis, inflammatory bowel disease, atherosclerosis, Alzheimer's and various cancer. Indonesian cassia (*Cinnamomum burmanni C. Nees & T. Ness*) known to contain coumarin, is widely used for alternative medicine especially as an antiinflammator. **The research objective** : This study was conducted to determine the anti-inflammatory properties of coumarin and Indonesian cassia extract (ICE) in LPS-induced RAW264.7 cell line. **Experimental approach**: The cytotoxic assay of coumarin and ICE against RAW264.7 cells was conducted using MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium). The anti-inflammatory potential was determined using LPS-induced RAW 267.4 macrophages cells to measure inhibitory activity of both compounds on production of **nitric oxide** (NO), prostaglandin E_2 (PGE_2), and also cytokines such as interleukin-6 (IL-6), interleukin-1β (IL-1β) and TNF-α. **Result** : Coumarin 10 **µM** and ICE 10 µg/ml were non toxic to the RAW264.7 cells. Both of coumarin and ICE were capable to reduce the PGE_2, TNF-α, NO, IL-6, and IL-β **level** in LPS-induced RAW264.7 cells. Coumarin had higher activity to decrease PGE_2 and TNF-α, whilst ICE had higher activity to inhibit NO, IL-6, and IL-β levels. **Conclusion**: Coumarin and ICE possess anti-inflammatory properties through inhibition of PGE_2 and NO along with pro-inflammatory cytokines TNF-α, IL-6, IL-1β production.

Keyword: Coumarin, Indonesian cassia extract, cytokines, RAW267.4, inflammation

INTRODUCTION

Inflammation is a biological response to tissue injury [1]. Inflammation relates with various diseases such as rheumatoid arthritis, inflammatory bowel disease, atherosclerosis, Alzheimer's, and has a role in various cancer development [2]. In inflammation, macrophage plays an important role by producing Reactive Oxygen Species (ROS), Reactive Nitrogen Species (RNS), cytokines such as **interleukin-1β (IL-1ß),** IL-6, **tumor necrosis factor-α** (TNF-α), and inflammatory mediator **nitric oxide (NO)** and prostaglandin (PGE). Exposure of bacterial lipopolysaccharides (LPS) has been found to increase the mRNA expression of those inflammatory cytokines and mediators [3, 4]. LPS is an bacterial endotoxin which stimulates innate immunity by regulating inflammatory mediator such as TNF-α, IL-6, and NO [5]. The suppression of inflammatory mediator synthesis has been known to be one of useful therapeutic strategy in the treatment of inflammatory diseases.

Recently, utilization of compounds isolated from herbal medicine for the treatment of inflammatory diseases has been gaining interest. This is due to in addition of their pleiotropic immune modulatory properties, they also had several other properties such as able to scavenge free radicals, non-toxic, and pharmacologically safe to use [4,8,9].

Indonesian cassia (*Cinnamomum burmanni (C. Nees & T. Ness*)) is one of plant which possess medicinal properties, for many years it has been widely used for treating dyspepsia, gastritis, and inflammatory diseases [10]. Indonesian cassia extract (ICE) has several constituents including cinnamic aldehyde, cinamic alcohol, cinnamic acid, coumarin, and carragean [6]. ICE has been shown to have many pharmacological activities such as anti-inflammatory, antipyretic, antimicrobial, antidiabetic and antitumor activity [11,12]. Coumarin, which is one of major compound in *C. burmannii*, is the plant derivate which possess anti-inflammatory and cancer chemo preventive properties [13]. Coumarin can reduce tissue edema and in inflammation it is an inhibitor of prostaglandin biosynthesis, which involves fatty acid hydroperoxy intermediates. It is to be expected that coumarin might affect the formation and scavenging of ROS and influence processes involving free radical-mediated injury [14]. The aim of this study was to analyze the anti-inflammatory activity of coumarin and **ICE** on the *in vitro* production of **inflammatory mediators** such as NO, PGE2 and cytokines IL-6, IL-1β and TNF-α.

MATERIALS AND METHODS

Extract preparation

Extraction was done based on the maceration method, Indonesian cassia (*Cinnamomum burmanni* (C. Nees & T. Ness)) bark was collected from Lembang, Bandung, West Java, Indonesia. **The plants were identified by herbarium staff, Department of Biology, School of Life Science and Technology, Bandung Institute of Technology, Bandung, West Java, Indonesia.** The dried skin of bark of Indonesian cassia (400 g) was milled and immersed in distilated ethanol 70%. After 24 h, the filtrates were collected and the residues were immersed again in 70% distilated ethanol for 24 h. **These treatments were repeated until the filtrate became colorless.** The collected filtrates were evaporated with a rotary evaporator at 40°C. The extracts were stored at -20°C **[15, 16]. The coumarin was used as standard and purchased from Chengdu Biopurify Phytochemical Ltd [91-64-5].**

Cell Culture

RAW264.7 macrophages cell line (ATCC ® TIB-71™) **was obtained by Biomolecular and Biomedical Research Center, Aretha Medika Utama. Bandung, Indonesia**. Cells were grown in **Dulbecco's Modified Eagle Medium (DMEM, Biowest L0060)** supplemented with contain **10% Fetal Bovine Serum (FBS) and 100 U/ml penicillin-streptomycin (Biowest L0022-100), and then incubated at 37ºC and 5% CO_2 until the cells were confluent. The cells then washed and harvested using trypsin-EDTA (Biowest, L0931-500) [17, 18].**

RAW264.7 Cells Viability Assay

The RAW264.7 cells viability was measured by MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay, based on the conversion of yellow tetrazolium salt to form a purple formazan product. MTS (Promega, Madison, WI,

USA) was used to determine the cytotoxicity of coumarin and ICE towards RAW264.7 cells. Cells were seeded in 96 well-plate (5,000 cell/well) then incubated for 24 hours. **After 24 h, the cells were supplemented with 90 µl fresh medium along with 10 µl of ICE in various concentrations (10, 50, 100 µg/ml) and coumarin (10, 50 and 100 µM) and then incubated at 37ºC with CO_2 5% for 24 h. Untreated cells were served as a negative control. After 24 h of incubation, MTS was added into each well at a ratio 1 : 5.** The plate was incubated at 37ºC and CO_2 5% for 3 hours. The absorbance was measured at 490 nm **[17-19].**

Pro-inflammatory activation of RAW264.7 Cells

The activation of inflammatory condition of the macrophage cells was performed according to **Rusmana et al (2015) and Dewi et al (2015)**. The cells were plated in 6-well plate **($1x10^5$ cells/well)**, incubated for 24 h at 37ºC in a humidified atmosphere and CO_2 5%. The culture medium was discharged, then the cells were supplemented with 1600 µM fresh medium along with 200 µl extract or coumarin **solution** in several concentration based on the viability assay. Around 1-2 h following the addition of extract or compound, 200 µl of **LPS from *E-coli* [Sigma Aldrich, L2880]** (1 µg/ml) was added into each well and the plate was incubated for 24 h at 37ºC, humidified atmosphere, 5% CO_2. The medium then was taken for the next assay and centrifuged at 2000 g for 10 min. The supernatant was collected, stored at -79ºC for quantification of IL-6, IL-1β, TBF-α, NO, and PGE_2 concentration **[18, 19]**

IL-6 assay

IL-6 concentration was determined using kit BioLegend (LEGEND MAX™ Mouse IL-6 ELISA Kit with Pre-coated Plates). The plate was washed using wash buffer 300 µl four times, 50 µl matrixes C was added into the standard well and 50 µl assay buffer was added into the sample well. Briefly, 50 µl samples were introduced in sample well and 50 µl standard solution was added in a standard well, the plate then incubated in orbital shaker 200 ppm for 2 hour at room temperature. Accordingly, 100 µl *Mouse IL-6 detection antibody* was added and the plate was incubated again for 1 hour in orbital shaker 200 ppm. The solution then discharged and the plate was washed using 200 µl of wash buffer for 4 times. A hundred microlitre of *Avidin HRP* solution was added into each well, the plate was kept at room temperature for 30 min in orbital shaker 200 ppm. The plate was washed again for 5 times and 100 µl of *substrate solution F* was added followed by incubation for 10 min in the dark room. Subsequently, 100 µl of stop solution was added and absorbance was read by using **Multiskan GO Microplate Reader** at 450 nm **[18-20].**

PGE_2 Assay

PGE_2 concentration was measured using an RnD System kit (KGE 004B). Calibration diluent was added into the well, each 200 µl for blank, 150 µl for standard, and 150 µl for sample wells. Subsequently, 50 µl of primary antibody solution was added into each well except blank well, then incubated at room temperature for 1 hour in an orbital shaker. Approximately 50 µl of PGE *Conjugate* was added into standard and sample wells, then placed in an orbital shaker for 2 h at room temperature. Plates were washed using 400 µl wash buffer four times, then 200 µl of substrate solution was added into each well followed by incubation for 30 min in dark room. Stop solution (100µl) was added into each well, then the absorbance was measured using **Multiskan GO Microplate Reader** with wavelength at 450 nm **[19].**

IL-1β assay

The IL-1β concentration was determined according to the manufacture's instruction manual (Biolegend ELISA kit, 432601). The plate was washed four times with at least 300µl of Wash buffer then sealed, incubated for 1 hours. In the standard well, 50µl of Matrix C was added, whilst 50µl of assay buffer sample was added into the sample well. Subsequently, 50µL of standard solution and 50µl of sample added into the sample well. The mixture then washed four times after incubated for 2 hours in orbital shaker. Following washing procedure, 100 µl of detection antibody was added into each well. The mixture then washed four times after incubated for 1 hour in orbital shaker. Afterward, 100µl of Avidin-HRP solution was added into each well and the plate was incubated at room temperature for 30 minutes on orbital shaker. The plate washed again five times, then substrate solution F (100 µl) was added into each well, incubated for 10 minutes in the dark

condition. The reaction was stopped by adding 100µl stop solution. The absorbance was read at 450 nm using **Multiskan GO Microplate Reader [18, 19].**

TNF-α assay

TNF-α levels in supernatant were determined by ELISA technique according to the manufacture's instruction manual (Biolegend ELISA kit, 421701). A hundred microlitre of capture antibody solution added to each well in 96-well plate and incubated at 4°C overnight. The plate was washed four times using 300µl of Wash Buffer, then incubated for one hour in shaker. Around 50 µl of Matrix C and 50 µl of Assay Buffer was added into each standard and sample well, respectively. Plates were shaked at room temperature and then washed for 4 times. Afterward, 100µl of the detection antibody solution was added into each well, incubated at room temperature for 1 hour on the orbital shaker. The plate then was washed four times. Subsequently, 100µl of diluted Avidin-HRP solution was added into each well, incubated at room temperature for 30 minutes in orbital shaker. The plate was washed again 5 times, then added with 100µl of substrate solution, incubated for 10 minutes in the dark room. The reaction was stopped by adding 100µl of stop solution briefly, and the absorbance was measured by **Multiskan GO Microplate Reader** at 450 nm **[18,19, 21].**

NO assay

The nitrite associated with NO production was determined using Abnova Kit (No cat. KA 1342) protocol. Briefly, 200 µl of assay buffer, 100 µl of standard solution, and 100 µl of sample was added into the blank, standard, and sample well, respectively. Around 50 µl of R1 and 50 µl of R2 then added into each well except for the blank well. The plate then incubated for 10 min at the room temperature, and absorbance was read at 540 nm using **Multiskan GO Microplate Reader [18, 19].**

RESULTS

Viability assay

Cell viability was measured by MTS assay based on the conversion of yellow tetrazolium salt to form a purple formazan product. Table 1 and 2 showed that the viability of RAW264.7 cells were over 90% in all treatments compared to the control (RAW264.7 cells without treatment), indicated that the coumarin and ICE in the concentration used were non toxic to the cells and can be applied for the next assay. Both coumarin **10 µM** and ICE **10 µg/ml** showed the highest cell viability.

IL-6 assay

Based on **Table 3**, it can be seen that both ICE and coumarin in concentration of 10 and 50 **µM, µg/ml** were able to inhibit IL-6 production in LPS-induced RAW264.7 cells. The LPS induction was successfully increase the IL-6 concentration, showed by significantly high IL-6 level in positive control (LPS-induced RAW264.7 cells without treatment) compared to the negative control (normal RAW264.7 cells without LPS induction). The ICE treatment showed higher IL-6 inhibition activity compared to the coumarin, and the IL-6 level was not differ significantly with the negative control, demonstrated its remarkable IL-6 inhibition properties.

PGE_2 assay

Quantification of PGE_2 revealed that ICE and coumarin had inhibition effect toward production of PGE_2 in LPS-induced RAW264.7 cells in concentration-dependent manner **(Table 4).** Among the treatments, coumarin 50 **µM** had the highest PGE2 inhibition activity whilst ICE 10 µg/ml showed the lowest activity. Coumarin in both concentration used (10, 50 **µM**) had significantly lower PGE_2 concentration compared to the ICE (10, 50 µg/ml), and even lower than the negative control. This suggested that coumarin had great abilities to inhibit PGE_2 production in inflammation condition.

IL-1β assay

Measurement of IL-1β levels of ICE **10, 50 µg/ml** and coumarin 10, 50 **µM treatment** revealed that the ICE treatments were succeeded in lowering IL-1β levels compared to the positive control. The coumarin treatments, however, were failed to reduce the IL-1β levels, showed by low IL-1β

inhibition activity over positive control **(Table 5).** Based on these results, it suggested that coumarin was not effective in inhibiting the production of pro-inflammatory cytokines IL-1β in LPS-induced RAW264.7 cells.

TNF-α assay

The examination of ICE and coumarin effect toward production of TNF-α revealed that both treatments were able to dose-dependently reduce TNF-α concentration in LPS-induced RAW264.7 cells, showed by TNF-α inhibition activity over positive control values in **Table 6.** The LPS induction was succeed in increasing TNF-α levels, as seen in the Table 6 the positive control (cell with LPS induction) had significantly higher TNF-α concentration than negative control (cell without LPS induction). In terms of inhibitory activity against TNF-α production, coumarin had significantly higher inhibition activity compared to ICE.

NO assay

The quantification of NO levels suggested that ICE **in concentration of 10, 50 µg/ml** and coumarin **10, 50 µM** were also able to inhibit the production of NO **(Table 7).** These were proved by decreasing levels of NO in LPS-induced cells treated with either ICE or coumarin compared to the positive control, which is untreated LPS-induced cells. The inhibition activity was found to be dose-dependent, and it can be clearly seen that coumarin had lower NO inhibition activity over positive control compared to ICE treatments. The negative control had the lowest NO level and the positive control had the highest NO level, indicating that LPS succeed to significantly increase NO concentration in RAW264.7 cells.

DISCUSSION

Several studies have been demonstrated that various compounds from plants possess rich pharmacological properties that play beneficial roles in many different diseases, including inflammation-related diseases [22]. Inflammation is a dynamic process involving proinflammatory cytokines, and it acts as important biological response toward injury [23, 24]. In this study, we examined the anti-inflammatory properties of ICE and coumarin using RAW264.7 murine macrophage cell line which has been widely used as an inflammatory model in vitro [24]. Coumarin (2H-1-benzopyran-2-one) is a component of natural materials which exhibit a variety of theurapeutical activities such as antiinflammation, anticoagulants, antibacterial, antifungal, anticancer, antihypertensive, antiadipogenik, antihiperglikemia and neuroprotective. Coumarin found in the oil of cinnamon rod (cinnamon bark oil), cassia oil and lavender oil, **Indonesia cassia. C. *burmanni* has anti-inflammatory** activity and safe for consumption in the long term [25].

Based on this study, coumarin and ICE may have potential to be used as anti-inflammatory agent to prevent chronic disease related to inflammation and did not possess toxicity effect toward RAW264.7 murine macrophage cell line shown in the viability assay. These results were supported by Arora et al. study [26], which reported that coumarin derivatives had antiinflammatory and antioxidant activity without side effect on gastric mucosa, furthermore it did not induce oxidative stress in tissues and sufficiently bioavailable.

Cytokines and mediator are produced by macrophage during the inflammatory process [19]. IL-6 has a wide range effect on cells of the immune system and its potent ability to induce the response due to acute inflammation [27]. IL-6 takes a part in hematopoiesis, immune response regulation, and inflammation. It has been reported that there was an increase of IL-6 level in the rheumatoid arthritis, psoriasis, and encephalomyelitis individuals [28], therefore inhibition of IL-6 synthesis would be useful for autoimmune disease and inflammation treatment. In the inflammation, IL-1β induces fever and secretion of IL-6 and IL-8 which are play a role as pro-inflammatory cytokines [23, 29]. Moreover, IL-1β is important for the initiation and increase of the inflammatory response to microbial infection [30]. In this study, coumarin and ICE could inhibit IL-6 and IL-1β production in RAW264.7 cell lines which suggest they have anti-inflammatory effect through down regulation of those pro-inflammatory cytokines. **Previous study showed that the better anti-inflammatory activities of coumarin isolated from *Glycirrhizae radix* that decrease mRNA expression of pro-**

inflammatory cytokines IL-1ß by 53.9% at 50 µM, IL-6 by 24.43% at 5 µM and 24.32% at 50 µM, and NO inhibition by 87.1% at 50 µM, in LPS stimulated RAW 267.4 cells [31]. Also, the newly isolated coumarin derivative (8-methoxy-chromen-2-one/MCO) from *Ruta graveolens* (Rue) plant in the collagen-induced arthritic (CIA) rat model showed inhibition of cytokines and NF-kB in LPS stimulated J774 cells [32].

The result of present study showed decrease of IL-6 and IL-β by ICE was higher than coumarin, that indicates other compounds content in plants to play its role in anti-inflammatory activities. Referring to previous phytochemical analysis on seven plants of the *Cinnamomum* species including *C. burmannii*, it showed four chemical constituents; cinnamaldehyde, cinnamic acid, cinnamyl alcohol, and coumarin, using RP-HPLC [33]. These compounds are suggested to work synergistically in anti-inflammatory activities.

NO and PGE_2 play critical roles in the aggravation of chronic inflammatory diseases, such as hepatic dysfunction and pulmonary disease. Nitric oxide (NO) play an important role in a variety of physiological and pathological processes including inflammatory reaction, thus NO has a potential theurapeutic implication inhibition in inflammation [34]. Recently, *in vitro* and *in vivo* studies have indicated an existing cross talk between the release of NO and prostaglandins (PGs) in the modulation of molecular mechanisms that regulate PGs generating pathway [35]. Scientific papers observed that while the production of both NO and PGE_2 was blocked by the NOS inhibitors in mouse macrophages RAW264.7 cells, these inhibitory effects were reversed by co-incubation with the precursor of NO synthesis, L-Arginine. Furthermore, inhibition of iNOS activity by nonselective NOS inhibitors attenuated the release of NO and PGs simultaneously in LPS activated macrophages [36]. Our present study showed that coumarin and ICE could decrease PGE_2 and NO level, which indicate that they have potential as anti-inflammatory. **The result of present study showed stronger activity than other study of *Cinnamomum cassia* that exhibited PGE_2 inhibition in LPS-stimulated RAW 264.7 cell (IC_{50} = 37.67±0.58 µM) [37].** Coumarin also reported could reduce tissue edema and inflammation, by inhibited prostaglandin biosynthesis which involve fatty acid hydroperoxy intermediates [23]. Coumarin of *Angelicae dahuricae* (CAD) could significantly lower PGE_2 levels in the inflammatory tissues, and its mechanism may be related to its inhibition of COX-2 expression which can cause the reduction of PGE_2 biosynthesis [38].

The other cytokine which play significant role in inflammation is TNF-α. TNF-α is an important cytokine that involved in inflammatory response by activating nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), cytokine, and adhesion molecule inducer [39-41, 18]. TNF-α inhibitory activity measurement is important in anti-inflammatory potential agent screening since this cytokine is an important mediator of inflammation [42]. This research showed that coumarin and ICE also can inhibit pro-inflammatory produced cytokines of TNF-α, although it was not comparable to negative control. **However, the result of the present study showed more activies than previous study, coumarin isolated from *Glycirrhizae radix* increased TNF-α mRNA expression instead [31].**

CONCLUSION

Coumarin and Indonesian cassia extract (ICE) possess anti-inflammatory activity showed by significantly decrease the production of pro-inflammatory mediators NO and PGE_2 level, also pro-inflammatory cytokines IL-6, IL-1β and TNF-α level in activated RAW264.7 macrophages.

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CONFLICTS OF INTEREST

All contributing authors declare no conflicts of interest

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Table 1. Mean, standard deviation and Duncan Post Test the absorbance of RAW264.7 cells using MTS assay (the research was done in triplicate)

Samples	Absorbance		
	Concentration (10 µg/ml **or µM**)	Concentration (50 µg/ml **or µM**)	Concentration (100 µg/ml **or µM**)
Control	0.7551±0.0252		
ICE **(µg/ml)**	0.7108±0.0071[a]	0.6996±0.0710[a]	0.7039±0.0185[a]
Coumarin **(µM)**	0.7849±0.0149[a]	0.7290±0.0194[b]	0.6912±0.0028[c]

Data are presented as mean ± standard deviation. Different superscript letters ([a-c]) in the same row are significant differences among the means of gropus (concentrations of ICE for the second row and concentrations of coumarin for the third row) based on Duncan post hoc test ($p < 0.05$)

Table 2. Mean, standard deviation and Duncan Post Hoc Test the viability of RAW264.7 cells over control (the research was done in triplicate)

Samples	Cell viablity (%)		
	Concentration (10 µg/ml **or µM**)	Concentration (50 µg/ml **or µM**)	Concentration (100 µg/ml **or µM**)
Control	100±3.33		
ICE **(µg/ml)**	94.14±0.93[a]	92.66±9.40[a]	93.22±2.45[a]
Coumarin **(µM)**	103.95±1.08[a]	96.55±2.57[b]	91.54±0.36[c]

Data are presented as mean ± standard deviation. Different superscript letters ([a-c]) in the same row are significant differences among the means of gropus (concentrations of ICE for the second row and concentrations of coumarin for the third row) based on Duncan post hoc test ($p < 0.05$)

Table 3. Mean, standard deviation and Duncan Post Hoc Test IL-6 detection (Il-6 level, IL-6 producing inhibition over positive control, IL-6 producing inhibition over negative control) [the research was done in triplicate]

Samples	IL-6 detection		
	IL-6 level (pg/ml)	IL-6 inhibition activity over positive control (%)	IL-6 inhibition activity over negative control (%)
Negative control	191.33±13.25[a]	60.58±2.73[d]	0.00±6.93[d]
Positive control	485.42± 11.56[d]	0.00±2.38[a]	-153.71±6.04[a]
ICE 50 µg/ml	191.00± 20.88[a]	60.65±4.30[d]	0.17±10.91[d]
ICE 10 µg/ml	190.58± 15.00[a]	60.74±3.09[d]	0.39±7.84[d]
Coumarin 50 **µM**	241.75± 11.66[b]	50.20±2.40[c]	-26.35±6.09[c]
Coumarin 10 **µM**	294.67± 18.07[c]	39.30±3.71[b]	-54.01±9.44[b]

***Data are presented as mean ± standard deviation. Inhibitory activity over positive control was measured based on comparison between treatment and positive control (positive control-treatment/positive control*100%), whilst inhibitory activity over negative control was measured based on comparison between treatment and negative control (negative control-treatment/negative control*100%). Different superscript letters ($^{a-d}$) in the same coloumn of IL-6 level, $^{a-d}$ of IL-6 inhibition activity over positive control, $^{a-d}$ IL-6 inhibition activity over negative control are significant differences among treatments based on Duncan pos hoc test with $p < 0.05$.**

Table 4. Mean, standard deviation and Duncan Post Hoc Test PGE_2 detection (PGE_2 level, PGE_2 producing inhibition over positive control, PGE_2 producing inhibition over negative control) [the research was done in triplicate]

Samples	PGE_2 detection		
	PGE_2 **level** (pg/ml)	PGE_2 inhibition activity over positive control (%)	PGE_2 inhibition activity over negative control (%)
Negative control	1,905.33±44.76^{c}	39.01±1.43^{c}	0.00±2.35^{d}
Positive control	3,124.00±70.02^{f}	0.00±2.24^{a}	-63.96±3.67^{a}
ICE 50 µg/ml	2,347.17±50.44^{d}	24.87±1.61^{c}	-23.19±2.65^{c}
ICE 10 µg/ml	2,773.50±46.87^{e}	11.22±1.50^{b}	-45.57±2.46^{b}
Coumarin 50 **µM**	1,648.67±26.04^{a}	47.23±0.83^{e}	13.47±1.37^{f}
Coumarin 10 **µM**	1,846.17±21.20^{b}	40.90±0.68^{d}	3.11±1.11^{e}

***Data are presented as mean ± standard deviation. Inhibitory activity over positive control was measured based on comparison between treatment and positive control (positive control-treatment/positive control*100%), whilst inhibitory activity over negative control was measured based on comparison between treatment and negative control (negative control-treatment/negative control*100%). Different superscript letters ($^{a-f}$) in the same coloumn of PGE2 level, $^{a-e}$ of PGE2 inhibition activity over positive control, $^{a-f}$ PGE2 inhibition activity over negative control are significant differences among treatments based on Duncan pos hoc test with $p < 0.05$**

Table 5. Mean, **standard deviation and** Duncan Post Hoc Test IL-1β (IL-1β level, IL-1β producing inhibition over postive control, IL-1β producing inhibition over negative control) ,

Samples	IL-1β **detection**		
	IL-1β level (pg/ml)	IL-1β inhibition activity over positive control (%)	IL-1β inhibition activity over negative control (%)
Negative control	794.68± 5.32^{a}	36.20± 0.43^{e}	0.00± 0.67^{e}
Positive control	1245.67± 3.52^{e}	0.00± 0.28^{a}	-56.75± 0.44^{a}
ICE 50 µg/ml	869.17± 7.45^{b}	30.22± 0.60^{d}	-9.37± 0.94^{d}
ICE 10 µg/ml	970.59± 3.22^{c}	22.08± 0.26^{c}	-22.14± 0.40^{c}
Coumarine 50 **µM**	1172.48± 48.72^{d}	5.88± 3.91^{b}	-47.54± 6.13^{b}
Coumarine 10 **µM**	1239.02± 1.04^{e}	0.53± 0.08^{a}	-55.91± 0.13^{a}

***Data are presented as mean ± standard deviation. Inhibitory activity over positive control was measured based on comparison between treatment and positive control (positive control-treatment/positive control*100%), whilst inhibitory activity over negative control was measured based on comparison between treatment and negative control (negative control-treatment/negative control*100%). Different superscript letters ($^{a-e}$) in the same coloumn of IL-1β level, IL-1β inhibition activity over positive control, IL-1β inhibition activity over negative control are significant differences among treatments based on Duncan pos hoc test with $p < 0.05$**

Table 6. Mean, **standard deviation and** Duncan Post Hoc Test **TNF-α** (**TNF-α** level, **TNF-α** producing inhibition over postive control, **TNF-α** producing inhibition over negative control)

Samples	**TNF-α detection**		
	TNF-α level (pg/ml)	**TNF-α** inhibition activity over positive control (%)	**TNF-α** inhibition activity over negative control (%)
Negative control	238.07±3.64[a]	46.88± 0.81[f]	0.00±1.53[e]
Positive control	448.13±20.08[f]	0.00± 4.48[a]	-88.24±8.44[a]
ICE 50 µg/ml	303.17±7.55[c]	32.35± 1.69[d]	-27.34±3.17[d]
ICE 10 µg/ml	368.39±15.41[e]	17.79± 3.44[b]	-54.74±6.47[b]
Coumarine 50 **µM**	260.43±5.34[b]	41.88± 1.19[e]	-9.39±2.24[d]
Coumarine 10 **µM**	321.47±8.81[d]	28.26± 1.97[c]	-35.03±3.70[c]

***Data are presented as mean ± standard deviation. Inhibitory activity over positive control was measured based on comparison between treatment and positive control (positive control-treatment/positive control*100%), whilst inhibitory activity over negative control was measured based on comparison between treatment and negative control (negative control-treatment/negative control*100%). Different superscript letters ([a-f]) in the same coloumn of TNF-α level, [a-f] of TNF-α inhibition activity over positive control, [a-d] TNF-α inhibition activity over negative control are significant differences among treatments based on Duncan pos hoc test with p < 0.05**

Table 7. Mean, **standard deviation and** Duncan Post Hoc Test NO (NO level, NO producing inhibition over postive control, NO producing inhibition over negative control)

Samples	NO **detection**		
	NO level (µM/ml)	NO inhibition activity over positive control (%)	NO inhibition activity over negative control (%)
Negative control	5.27±0.04[a]	85.27±0.10[f]	-0.07±0.69[f]
Positive control	35.79±0.25[f]	0.01±0.71[a]	-578.94±4.84[a]
ICE 50 µg/ml	17.04±0.09[b]	52.39±0.25[e]	-223.30±1.70[e]
ICE 10 µg/ml	21.63±0.13[c]	39.55±0.37[d]	-310.45±2.48[d]
Coumarine 50 **µM**	22.24±0.16[d]	37.85±0.46[c]	-322.00±3.12[c]
Coumarine 10 **µM**	31.73±0.35[e]	11.33±0.98[b]	-502.07±6.65[b]

***Data are presented as mean ± standard deviation. Inhibitory activity over positive control was measured based on comparison between treatment and positive control (positive control-treatment/positive control*100%), whilst inhibitory activity over negative control was measured based on comparison between treatment and negative control (negative control-treatment/negative control*100%). Different superscript letters ([a-f]) in the same coloumn of NO level, [a-f] NO inhibition activity over positive control, [a-f] NO inhibition activity over negative control are significant differences among treatments based on Duncan pos hoc test with p < 0.05**

Should be:	
Italic	*In vitro- In vivo*
Only the first letter of the title should be capitalized. Other letters should be typed in lower case. Also within the tables and figures. DO NOT USED ABBREVIATIONS IN TITLES. No frame or background or extra lines.	Title of Figures and Tables
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