Iranian Journal of Basic Medical Sciences

ijbms.mums.ac.ir

IJBMS

In vitro assesment of anti-inflammatory activities of coumarin and Indonesian cassia extract in RAW264.7 murine macrophage cell line

Ni Made Dwi Sandhiutami [1], Moordiani Moordiani [1], Dian Ratih Laksmitawati [1], Nurul Fauziah [2], Maesaroh Maesaroh [2], Wahyu Widowati [3*]

[1] Faculty of Pharmacy, University of Pancasila, Jl. Srenseng Sawah, Jagakarsa, Jakarta 12640, Indonesia
[2] Bimolecular and Biomedical Research Center, Aretha Medika Utama,Jl. Babakan Jeruk 2 no 9, Bandung 40163, Indonesia
[3] Medical Research Center, Faculty of Medicine, Maranatha Christian University, Jl. Prof. drg. Suria Sumantri no 65 Bandung 40164, Indonesia

ARTICLE INFO

Article type:
Short communication

Article history:
Received: Mar 31, 2016
Accepted: Oct 18, 2016

Keywords:
Coumarin
Cytokines
Indonesian cassia extract
Inflammation
RAW264.7

ABSTRACT

Objective(s): Inflammation is an immune response toward injuries. Although inflammation is healing response, but in some condition it will lead to chronic disease such as rheumatoid arthritis, inflammatory bowel disease, atherosclerosis, Alzheimer's and various cancer. Indonesian cassia (*Cinnamomum burmannil* C. Nees & T. Ness) known to contain coumarin, is widely used for alternative medicine especially as an anti-inflammatory. This study was conducted to determine the anti-inflammatory properties of coumarin and Indonesian cassia extract (ICE) in LPS-induced RAW264.7 cell line.

Materials and Methods: 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 compound and ISEon production of nitric oxide (NO), prostaglandin E2 (PGE2), and also cytokines such as interleukin-6 (IL-6), interleukin-1β (IL-1β) and TNF-α.

Results: Coumarin 10 µM and ICE 10 µg/ml were nontoxic to the RAW264.7 cells. Both of coumarin and ICE were capable to reduce the PGE2, TNF-α, NO, IL-6, and IL-β level in LPS-induced RAW264.7 cells. Coumarin had higher activity to decrease PGE2 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 PGE2 and NO along with pro-inflammatory cytokines TNF-α, IL-6, IL-1β production.

► *Please cite this article as:*
Sandhiutami NMD, Moordiani M, Laksmitawati DR, Fauziah N, Maesaroh M, Widowati W. *In vitro* assesment of anti-inflammatory activities of coumarin and Indonesian cassia extract in RAW264.7 murine macrophage cell line. Iran J Basic Med Sci 2017; 20:99-106; http://dx.doi.org/10.22038/ijbms.2017.8102

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 developments (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 a 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 the 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 immunemodulatory 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 burmannil* C. Nees & T. Ness) is one of plants 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

*Corresponding author: Wahyu Widowati. Medical Research Center, Faculty of Medicine, Maranatha Christian University, Bandung, Indonesia. Tel: +6281910040010; email: wahyu_w60@yahoo.com

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, one of major compounds 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 (*C. burmannil* 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 distilled ethanol 70%. After 24 hr, the filtrates were collected and the residues were immersed again in 70% distilled ethanol for 24 hr. 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 from 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 1% penicillin-streptomycin (Biowest L0022-100), and then incubated at 37 ˚C and 5% CO_2 until the cells were confluent. The cells then washed and harvested using trypsin-EDTA (Biowest, L0931-500) (17-19).

RAW264.7 cells viability assay

The RAW264.7 cells viability was measured by MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-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 toward RAW264.7 cells. Cells were seeded in 96 well-plates (5,000 cell/well) then incubated for 24 hr. After 24 hr, 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 37 °C with CO_2 5% for 24 hr. Untreated cells were served as a negative control. After 24 hr of incubation, MTS was added into each well at a ratio 1: 5. The plate was incubated at 37 °C and CO2 5% for 3 hr. 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), Dewi et al (2015), Widowati *et al* (2016) and Zhu *et al* (2013). The cells were plated in 6-well plate (5x105 cells/well), incubated for 24 hr at 37 °C in a humidified atmosphere and CO_2 5%. The culture medium was discharged, then the cells were supplemented with 1,600 µM fresh medium along with 200 µl extract or coumarin solution in several concentration based on the viability assay. Around 1-2 hr following the addition of extract or compound, 200 µl of LPS from *Escherichia coli* [Sigma Aldrich, L2880] (1 µg/ml) was added into each well and the plate was incubated for 24 hr at 37 ˚C, humidified atmosphere, 5% CO_2. The medium then was taken for the next assay and centrifuged at 2000xg for 10 minute. The supernatant was collected, stored at -79 ˚C for quantification of IL-6, IL-1β, TNF-α, NO, and PGE_2 concentration (17-20).

IL-6 assay

IL-6 concentration was determined using kit BioLegend 431301 (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 solutions was added in a standard well, the plate then incubated in orbital shaker 200 ppm for 2 hr at room temperature. Accordingly, 100 µl Mouse IL-6 detection antibody was added and the plate was incubated again for 1 hr 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 (18-21).

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 hr 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 hr 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 (100 µl) was added into each well, then the absorbance was measured using Multiskan GO Microplate Reader with wavelength at 450 nm (20).

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 300 µl of wash buffer then sealed, incubated for 1 hr. In the standard well, 50 µl of matrix C was added, whilst 50 µl of assay buffer sample was added into the sample well. Subsequently, 50 µl of standard solution and 50 µl of sample added into the sample well. The mixture was washed four times after incubated for 2 hr in orbital shaker. Following washing procedure, 100 µl of detection antibody was added into each well. The mixture was washed four times after incubated for 1 hr in orbital shaker. Afterward, 100 µl of Avidin-HRP solution was added into each well and the plate was incubated at room temperature for 30 min on orbital shaker. The plate washed again five times, then substrate solution F (100 µl) was added into each well, incubated for 10 min in the dark condition. The reaction was stopped by adding 100 µl stop solution. The absorbance was read at 450 nm using Multiskan GO Microplate Reader (18-20).

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 300 µl 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 shaken at room temperature and then washed for 4 times. Afterward, 100 µl of the detection antibody solution was added into each well, incubated at room temperature for 1 hr on the orbital shaker. The plate then was washed four times. Subsequently, 100 µl of diluted Avidin-HRP solution was added into each well, incubated at room temperature for 30 min in orbital shaker. The plate was washed again 5 times, then added with 100 µl of substrate solution, incubated for 10 min in the dark room. The reaction was stopped by adding 100 µl of stop solution briefly, and the absorbance was measured by Multiskan GO Microplate Reader at 450 nm (18, 20, 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 (18-20).

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 nontoxic 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 µg/ml, µM were able to inhibit IL-6 production in LPS-induced RAW264.7 cells. The LPS induction was successfully

Table 1. Mean, standard deviation and Duncan *post-hoc* test of the absorbance of RAW264.7 cells

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 groups (concentrations of ICE for the second row and concentrations of coumarin for the third row) based on Duncan *post-hoc* test (P-value < 0.05); ICE: Indonesian cassia extract

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 groups (concentrations of ICE for the second row and concentrations of coumarin for the third row) based on Duncan *post-hoc* test (*P*-value < 0.05); ICE: Indonesian cassia extract

Table 3. Mean, standard deviation and Duncan *post-hoc* test of interleukin-6 (level, inhibition over positive control, inhibition over negative control)

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*-value < 0.05); ICE: Indonesian cassia extract

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 different 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

Table 4. Mean, standard deviation and Duncan *post-hoc* test of Prostglandin E2 (level, inhibition over positive control, inhibition over negative control)

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] of PGE2 inhibition activity over negative control are significant differences among treatments based on Duncan *pos-hoc* test with (*P*-value<0.05); ICE: Indonesian cassia extract

Table 5. Mean, standard deviation and Duncan *post-hoc* test of interleukin-1β (level, inhibition over postive control, 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, [a-e] of IL-1β inhibition activity over positive control, [a-e] of IL-1β inhibition activity over negative control are significant differences among treatments based on Duncan *pos-hoc* test with (*P*-value<0.05); ICE: Indonesian cassia extract

Table 6. Mean, standard deviation and Duncan *post-hoc* test of tumor necrosis factor-α (level, inhibition over postive control, 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] of TNF-α inhibition activity over negative control are significant differences among treatments based on Duncan *pos-hoc* test with (*P*-value<0.05); ICE: Indonesian cassia extract

Table 7. Mean, standard deviation and Duncan *post-hoc* test of nitrite oxide (level, inhibition over postive control, 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] of NO inhibition activity over positive control, [a-f] of NO inhibition activity over negative control are significant differences among treatments based on Duncan pos-hoc test with (*P*-value<0.05); ICE: Indonesian cassia extract

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 treatments revealed that the ICE treatments were succeeded in lowering IL-1β levels compared to the positive control. The coumarin treatments, however, 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, shown 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 (23). Inflammation is a dynamic process involving proinflammatory cytokines, and it acts as important biological response toward injury (24-25). 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* (17-19, 25). Coumarin (2H-1-benzopyran-2-one) is a

component of natural compounds which exhibit a variety of therapeutic activities such as antiinflammation, anticoagulant, antibacterial, antifungal, anticancer, antihypertensive, antiadipogenic antihyperglycemic and neuroprotective. Coumarin found in the oil of Cinnamon rod (cinnamon bark oil), cassia oil and lavender oil, Indonesian cassia. C. *burmannil* has anti-inflammatory activity and safe for consumption in the long term (26).

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 (27), which reported that coumarin derivatives had anti-inflammatory 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 (20). 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 (28). 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 (29), 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 (24, 30). Moreover, IL-1β is important for the initiation and increase of the inflammatory response to microbial infection (31). 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 (32). 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 (33).

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 (34). These compounds are suggested to work synergistically in anti-inflammatory activities.

Nitric oxide (NO) and PGE_2 play critical roles in the aggravation of chronic inflammatory diseases, such as hepatic dysfunction and pulmonary disease. 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 (35). 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 (36). 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 (37). 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 C. cassia that exhibited PGE2 inhibition in LPS-stimulated RAW 264.7 cell (IC_{50}=37.67±0.58 μM) (38). Coumarin also reported could reduce tissue edema and inflammation, by inhibited prostaglandin biosynthesis which involve fatty acid hydroperoxy intermediates (24). Coumarin of Angelicae dahuricae (CAD) could significantly lower PGE2 levels in the inflammatory tissues, and its mechanism may be related to its inhibition of COX-2 expression which can cause the reduction of PGE2 biosynthesis (39).

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 (18, 40-42). TNF-α inhibitory activity measurement is important in anti-inflammatory potential agent screening since this cytokine is an important mediator of inflammation (43). This research showed that coumarin and ICE also can inhibit pro-inflammatory 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 (32).

Conclusion

Coumarin and Indonesian cassia extract (ICE) possess anti-inflammatory activity showed by significantly decrease in 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.

Acknowledgment

We gratefully acknowledge the financial support of the Directorate General of Higher Education, National Ministry of Republic Indonesia for research grant of Hibah Bersaing 2014. This study was succesfully done by the facilities support of Biomolecular and Biomedical Research Center, Aretha Medika Utama and valuable assistance by Merry Afni, Ervi Afifah, Hanna Sari W. Kusuma, Dwi Davidson Rihibiha, Hayatun Nufus from Biomolecular and Biomedical Research Center, Aretha Medika Utama, Bandung, West Java, Indonesia.

Conflict of interest

All contributing authors declare no conflicts of interest.

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