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journal homepage: www.elsevier.com/locate/apjtbOriginal article <https://doi.org/10.1016/j.apjtb.2017.10.002>Anti-inflammatory properties of oolong tea (*Camellia sinensis*) ethanol extract and epigallocatechin gallate in LPS-induced RAW 264.7 cellsArina Novilla¹, Dedi Somantri Djahuri¹, Betty Nurhayati², Dwi Davidson Rihibiha³, Ervi Afifah³, Wahyu Widowati^{4*}¹School of Health Sciences Jenderal Achmad Yani Cimahi, Jl. Terusan Jenderal Sudirman, Cimahi 40533, West Java, Indonesia²Department of Health Analyst, Health Sciences Polytechnic Bandung, Jl. Prof. Eyckman No.24, Bandung 40161, West Java, Indonesia³Biomolecular and Biomedical Research Center, Aretha Medika Utama, Jl. Babakan Jeruk 2 No. 9, Bandung 40163, West Java, Indonesia⁴Medical Research Center, Faculty of Medicine, Maranatha Christian University, Jl. Prof drg. Suria Sumantri No.65, Bandung 40164, West Java, Indonesia

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ABSTRACT

Objective: To evaluate the anti-inflammatory activity of oolong tea ethanol extract (OTEE) and epigallocatechin gallate (EGCG) on lipopolysaccharide-induced murine macrophage cell line (RAW 264.7).**Methods:** A cytotoxic assay using MTS tetrazolium was conducted to find a nontoxic level of OTEE and EGCG toward RAW 264.7 cells. Interleukins (IL-6, IL-1 β), tumor necrosis factor- α (TNF- α), and cyclooxygenase-2 (COX-2) levels were measured by ELISA, and nitric oxide (NO) levels measured by a nitrate/nitrite colorimetric assay to determine the inhibition activity of OTEE and EGCG.**Results:** Lipopolysaccharide induction increases NO, COX-2, IL-6, IL-1 β , and TNF- α levels compared with the untreated cell (negative control). The positive control, lipopolysaccharide-induced RAW 264.7 without treatments showed the highest level of all pro-inflammatory cytokines and modulators tested in this study. The positive control was used as standard to obtain OTEE and EGCG inhibition activity toward NO, COX-2, IL-6, IL-1 β , and TNF- α . OTEE had a higher inhibition activity toward NO, COX-2, IL-6, and IL-1 β than EGCG; the reverse was seen for TNF- α . However, both OTEE and EGCG suppressed production of NO, COX-2, IL-6, IL-1 β , and TNF- α .**Conclusions:** OTEE and EGCG have the potential for use as anti-inflammatory drugs, which is shown by their ability to reduce the production of NO, COX-2, IL-6, IL-1 β , and TNF- α in active macrophages.

1. Introduction

Inflammation is a complex process regulated by pro-inflammatory cytokines and mediators that occur as an innate

immune response to irritation and infection caused by pathogens, wounding, and chemicals. It is characterized by recruitment of a wide range of immune cells to the inflamed sites such as neutrophils, macrophages, and monocytes [1]. During inflammation, the primary cell of chronic inflammation, which is a macrophage, is activated by exposure to interferon- γ , pro-inflammatory cytokines, or bacterial lipopolysaccharide (LPS) [2]. Activated macrophage release amounts of inflammatory mediators such as nitric oxide (NO) and pro-inflammatory cytokines such as interleukin (IL-12, IL-1 β , IL-6), tumor necrosis factor alpha (TNF- α) [2]. TNF- α initiates and regulates inflammatory process at the multicellular level, with an

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expression of pro-inflammatory cytokines, e.g., IL-1 and IL-6 [3]. The excessive production of inflammatory mediators and cytokines in prolonged inflammation can cause cellular and tissue damage. NO overproduction leads to cellular response including apoptosis and necrosis [4]. In tissue level, inflammatory mediators and cytokines cause many pathophysiologic processes in the development of chronic diseases, some of which are cancer, diabetes, cardiovascular disease, atherosclerosis, and inflammatory arthritis [5].

To prevent the side effect of prolonged inflammation, anti-inflammatory agents are needed. Any substances that inhibit production of these pro-inflammatory molecules are considered as potential anti-inflammatory agents [6]. Today, many synthetic drugs are used extensively in order to avoid chronic inflammation. However, prolonged consumption of these drugs is sometimes coupled with their own side effects [7]. Naturally derived substances for preventing prolonged inflammation have limited side effects and fewer problems with intolerance, while these substances are available at lower costs than synthetic drugs [1].

Some of the most promising natural substances against chronic inflammation are the polyphenols. Polyphenols are found abundantly in tea (*Camellia sinensis*), and have been shown to have anti-inflammatory activity in suppressing the synthesis and action of many pro-inflammatory mediators. Theasinsensins, the primary polyphenols in oolong tea, are thought to potentially inhibit cyclooxygenase-2 (COX-2) expression in LPS-activated mouse macrophage-like cells (RAW 264.7) [8]. Epigallocatechin gallate (EGCG), found in green tea, also has anti-inflammatory activity through its ability to scavenge NO, peroxynitrite and other reactive oxygen and nitrogen species [4]. Accordingly, this study aims to evaluate anti-inflammatory activity of oolong tea ethanol extract (OTEE) and EGCG through assessing their effects on IL-6, IL-1 β , TNF- α , COX-2, and NO levels in an LPS-induced murine macrophage cell line (RAW 264.7) model.

2. Materials and methods

2.1. EGCG and oolong tea extraction

EGCG (purity 95%–99% by HPLC-DAD) was purchased from Biopurify Phytochemical Ltd. (Chengdu, China). Oolong tea (*Camellia sinensis*) was obtained from a tea plantation in East Java. Oolong tea was crushed into fine powder, and then extracted with 96% methanol using a maceration technique. The filtrate was filtered and collected every 24 h until the filtrate became colorless. The filtrate was evaporated at 40 °C in an evaporator until a dried pellet was obtained. The ethanol-extracted pellet was stored at 4 °C prior to use [9].

2.2. 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. RAW 264.7 cells were grown in DMEM (Dulbecco's Modified Eagle Medium; Biowest) supplemented with 10% fetal bovine serum (FBS; Biowest) and 1% antibiotic–antimycotic (Biowest). The cells were incubated at 37 °C and 5% CO₂ in humidified atmosphere until confluent (80%–90%). Trypsin–EDTA (Biowest) was used to harvest the cells which then seeded on plates for the assays [10,11].

2.3. OTEE and EGCG cytotoxicity assay

The cytotoxicity of OTEE and EGCG was evaluated by assessing the viability of RAW 264.7 cells by using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium assay (Promega, Madison, WI, USA). A total of 100 μ L of medium (DMEM supplemented with 10% FBS, and 1% antibiotic–antimycotic) containing around 5×10^3 RAW 264.7 cells was seeded in each well of a 96-well plate, which was then incubated for 24 h at 37 °C, 5% CO₂ in a humidified atmosphere. The medium was washed from the cells and the cells were then supplemented with 90 μ L of fresh medium and 10 μ L of OTEE (100, 50, and 10 μ g/mL) or EGCG (100, 50, and 10 μ M), and incubated for 24 h. To all of the wells, 20 μ L of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium was added and the plate was incubated at 37 °C, 5% CO₂ for 3 h. The absorbance was measured at 490 nm. Cells without treatment were served as control, and % viability was obtained from the difference in viable cells from each treatment from the control [9–11].

2.4. LPS-induced RAW 264.7

There are six treatments used for this research: (1) positive control, RAW 264.7 cells were induced inflammation using 1 μ g/mL lipopolysaccharide (LPS from *Escherichia coli*) (Sigma). (2) negative control RAW 264.7 cells without induced lipopolysaccharide. (3) RAW 264.7 cells were added 1 μ g/mL lipopolysaccharide and OTEE 50 μ g/mL. (4) RAW 264.7 cells were added 1 μ g/mL lipopolysaccharide and OTEE 10 μ g/mL. (5) RAW 264.7 cells were added 1 μ g/mL lipopolysaccharide and EGCG 50 μ M. (6) RAW 264.7 cells were added 1 μ g/mL lipopolysaccharide and EGCG 10 μ M. Then, the cells were incubated for 24 h. Content in each well was then centrifuged, and the cell-free supernatant was used for the IL-6, IL-1 β , COX-2, NO, and TNF- α assays [10,11].

2.5. NO level assay

Quantification of NO used a nitrate/nitrite colorimetric assay kit protocol (Abnova). The absorbance was measured at 540 nm using an ELISA reader, MultiSkán Go (Thermo Scientific). The inhibition activity of each OTEE and EGCG treatment toward NO was obtained from the percentage (%) of NO concentration in each treatment compared to the positive control [10,11].

2.6. COX-2 level assay

Quantification of COX-2 used a Mouse PTGS2/COX-2 ELISA kit protocol (Elabscience) and the absorbance was measured at 450 nm. The inhibition activity toward COX-2 was obtained from the percentage (%) of COX-2 concentration in each treatment compared to the positive control.

2.7. TNF- α level assay

Quantification of TNF- α used a Mouse TNF- α ELISA MAX[™] Standard kit protocol (BioLegend) and the absorbance was measured at 450 nm. The inhibition activity toward TNF- α was obtained from the percentage (%) of TNF- α concentration in each treatment compared to the positive control [10,11].

2.8. IL-6 level assay

Quantification of IL-6 used a LEGEND MAX™ Rat IL-6 ELISA kit protocol (BioLegend). The absorbance was measured at 450 nm using an ELISA reader. The inhibition activity toward IL-6 was obtained from the percentage (%) of IL-6 concentration in each treatment compared to the positive control.

2.9. IL-1β level assay

Quantification of IL-1β used a Mouse IL-1β ELISA MAX™ Standard kit protocol (BioLegend). The absorbance was measured at 450 nm using an ELISA reader. The inhibition activity toward IL-1β was obtained from the percentage (%) of IL-1β concentration in each treatment compared to the positive control [10,11].

2.10. Statistical analysis

SPSS software (version 17.00) was used to statistically analyze all the data. A one-way ANOVA was used for finding any significant difference between treatments, $P < 0.05$ was considered to be significant and further significance between groups was analyzed using a Duncan *post hoc* test. Results are presented as the mean ± standard deviation of 3 independent experiments.

3. Results

A cytotoxic assay as a preliminary study showed that more than 90% of RAW 264.7 cells were viable at 50 and 10 μg/mL of OTEE and at 50 and 10 μM of EGCG. OTEE and EGCG at higher levels, 100 μg/mL and 100 μM, showed the cytotoxic effect to the cells by reducing RAW 264.7 cell viability by 44.55% and 37.30%, respectively (Table 1). LPS induction increases NO, COX-2, IL-6, IL-1β, and TNF-α levels compared with the untreated cell (negative control). The positive control, LPS-induced RAW 264.7 without treatments showed the highest level of all pro-inflammatory cytokines and modulators tested in this study. The positive control was used as standard to obtain OTEE and EGCG inhibition activity toward NO, COX-2, IL-6, IL-1β, and TNF-α (Tables 2, 4–6).

OTEE and EGCG decreased all the pro-inflammatory cytokines and mediators tested in this study compared to each positive control, except for 10 μg/mL of OTEE which showed higher TNF-α level than its positive control. The 50 μg/mL OTEE treatment showed the highest NO and IL-6 inhibition activities with 30.95% and 56.69%, respectively (Tables 2 and 5), while

Table 1

RAW 264.7 viability toward various OTEE and EGCG concentration (mean ± sd) (n = 3).

Treatment	Cell viability (%)
OTEE (100 μg/mL)	55.45 ± 6.78
OTEE (50 μg/mL)	121.97 ± 18.32
OTEE (10 μg/mL)	208.28 ± 17.13
EGCG (100 μM)	62.70 ± 3.44
EGCG (50 μM)	96.93 ± 5.61
EGCG (10 μM)	150.09 ± 5.60

Table 2

NO level and NO inhibition activity of OTEE and EGCG over positive control (mean ± sd) (n = 3).

Treatment	NO level (pg/mL)	NO inhibition activity (%)
Positive control	33.97 ± 0.10 ^c	0.00 ± 0.31 ^a
Negative control	5.03 ± 0.08 ^{ab}	85.18 ± 0.22 ^c
OTEE (50 μg/mL)	23.46 ± 0.05 ^b	30.95 ± 0.15 ^d
OTEE (10 μg/mL)	23.96 ± 0.02 ^d	29.48 ± 0.07 ^b
EGCG (50 μM)	23.64 ± 0.06 ^c	30.42 ± 0.18 ^c
EGCG (10 μM)	23.71 ± 0.05 ^c	30.19 ± 0.15 ^c

Different superscript letters in the same column (among concentrations of OTEE, EGCG in NO level, NO inhibition activity) indicate a significant difference at $P < 0.05$ (Duncan *post hoc* test).

Table 3

COX-2 level and COX-2 inhibition activity of OTEE and EGCG over positive control (mean ± sd) (n = 3).

Treatment	COX-2 level (pg/mL)	COX-2 inhibition activity (%)
Positive control	2.62 ± 0.21 ^c	0.13 ± 7.95 ^a
Negative control	0.83 ± 0.09 ^a	68.45 ± 3.42 ^e
OTEE (50 μg/mL)	1.99 ± 0.24 ^d	24.17 ± 9.02 ^b
OTEE (10 μg/mL)	1.38 ± 0.08 ^b	47.46 ± 2.86 ^d
EGCG (50 μM)	1.61 ± 0.34 ^{bc}	38.68 ± 13.14 ^{cd}
EGCG (10 μM)	1.86 ± 0.01 ^{cd}	28.88 ± 0.44 ^{bc}

Different superscript letters in the same column (among concentrations of OTEE, EGCG in COX-2 level, COX-2 inhibition activity) indicate a significant difference at $P < 0.05$ (Duncan *post hoc* test).

Table 4

TNF-α level and TNF-α inhibition activity of OTEE and EGCG over positive control.

Treatment	TNF-α level (pg/mL)	TNF-α inhibition activity (%)
Positive control	469.97 ± 67.35 ^c	0.00 ± 14.34 ^a
Negative control	228.14 ± 11.29 ^a	51.49 ± 2.40 ^f
OTEE (50 μg/mL)	290.29 ± 10.85 ^{ab}	38.26 ± 2.31 ^{bc}
OTEE (10 μg/mL)	470.88 ± 13.13 ^c	-0.19 ± 2.79 ^a
EGCG (50 μM)	261.56 ± 80.86 ^a	44.37 ± 17.22 ^c
EGCG (10 μM)	373.57 ± 75.16 ^{bc}	20.53 ± 16.00 ^{ab}

Different superscript letters in the same column (among concentrations of OTEE, EGCG in TNF-α level, TNF-α inhibition activity) indicate a significant difference at $P < 0.05$ (Duncan *post hoc* test).

Table 5

IL-6 level and IL-6 inhibition activity of OTEE and EGCG over positive control (mean ± sd) (n = 3).

Treatment	IL-6 level (pg/mL)	IL-6 inhibition activity (%)
Positive control	574.71 ± 57.23 ^c	0.00 ± 9.96 ^a
Negative control	167.57 ± 27.60 ^a	70.84 ± 4.80 ^d
OTEE (50 μg/mL)	248.90 ± 17.80 ^{ab}	56.69 ± 3.10 ^{cd}
OTEE (10 μg/mL)	323.09 ± 62.89 ^c	43.78 ± 10.94 ^c
EGCG (50 μM)	327.67 ± 80.14 ^c	42.99 ± 13.95 ^c
EGCG (10 μM)	455.38 ± 26.35 ^d	20.76 ± 4.58 ^b

Different superscript letters in the same column (among concentrations of OTEE, EGCG in IL-6 level, IL-6 inhibition activity) indicate a significant difference at $P < 0.05$ (Duncan *post hoc* test).

Table 6

IL-1 β level and IL-1 β inhibition activity of OTEE and EGCG over positive control (mean \pm sd) ($n = 3$).

Sample	IL-1 β level (pg/mL)	IL-1 β inhibition activity (%)
Positive control	1 195.18 \pm 22.95 ^c	0.00 \pm 1.92 ^a
Negative control	853.03 \pm 24.10 ^a	28.63 \pm 2.02 ^d
OTEE (50 μ g/mL)	897.77 \pm 134.07 ^{ab}	24.88 \pm 11.22 ^{cd}
OTEE (10 μ g/mL)	854.67 \pm 41.52 ^a	28.49 \pm 3.47 ^d
EGCG (50 μ M)	1 005.98 \pm 40.02 ^{bc}	15.83 \pm 3.35 ^{bc}
EGCG (10 μ M)	1 101.62 \pm 48.07 ^{cd}	7.83 \pm 4.02 ^{ab}

Different superscript letters in the same column (among concentrations of OTEE, EGCG in IL-1 β level, IL-1 β inhibition activity) indicate a significant difference at $P < 0.05$ (Duncan *post hoc* test).

50 μ M of EGCG provided the highest TNF- α inhibition activity (44.37%) (Table 4). Lastly, 10 μ g/mL of OTEE showed the highest COX-2 and IL-1 β inhibition activity by 47.46% and 28.49%, respectively (Tables 3 and 6).

4. Discussion

As noted above, several studies have reported that oolong tea and tea polyphenols exerted biological effects including antioxidant, antimutagenic, anticancer, and anti-inflammatory. In this study, we evaluated the anti-inflammatory properties of OTEE and EGCG toward inhibition of TNF- α , IL-6, IL-1 β , COX-2, and NO production in LPS-induced mouse macrophage-like cells (RAW 264.7). The ability of OTEE to suppress pro-inflammatory cytokines and mediators is likely due to several active compounds, especially polyphenols. Previous studies have shown that theasinensin A, a major polyphenol in oolong tea, could suppress the expression of inflammatory mediators such as COX-2 and prostaglin E₂ by attenuating cellular signaling, including the mitogen-activated protein kinase and NF- κ B pathways [12]. Nagai *et al.* [4], using rat hippocampal neuron cells showed that EGCG, the main polyphenol present in green tea, inhibited NO production in a dose-dependent manner at concentrations ranging from 50 to 200 μ M, and also demonstrated that EGCG could protect against ischemic neuronal damage by deoxidizing peroxynitrite/peroxynitrate, which is converted to an NO or hydroxyl radical [4]. Moreover, EGCG has been shown to suppress NO production by inhibiting inducible nitric oxide synthase expression in LPS/cytokine-induced human chondrocytes and in LPS/cytokine-induced murine macrophages by blocking NF- κ B activation [13].

The RAW 264.7 murine macrophage cell line was used to generate an inflammation environment by inducing an inflammation response in these cells with LPS. LPS is an endotoxin and a component of the outer membrane of Gram-negative bacteria [14]. In macrophages or monocytes, LPS induces an inflammatory response by initiating signal transduction through toll-like receptor 4 to activate expression of pro-inflammatory cytokines and mediators, including NO, IL-1, IL-6, and TNF- α [12,15]. As seen in a positive control, LPS-induced RAW 264.7 resulted in a significant increase of TNF- α , IL-6, IL-1 β , COX-2, and NO compared to the negative control. To evade adverse effects to RAW 264.7 cells prior to the usage of OTEE and EGCG, a cytotoxic assay was conducted. The result showed OTEE (50 and 10 μ g/mL) and EGCG (50 and 10 μ M) were safe for the RAW 264.7 cell growth.

At the multicellular level, TNF- α coordinates the inflammatory process by up-regulating other pro-inflammatory cytokines (e.g., IL-6, IL-1), inducing angiogenesis, activating transcription factor NF- κ B, and stimulating NO production [16]. Because of its multiple roles in inflammation, TNF- α has been targeted for screening as an anti-inflammatory agent [17]. IL-6 and IL-1 β are synthesized mainly by macrophages and have their own activities and effects in inflammation. IL-6 activates neutrophils and NK-cells [18], plays a role in the acute-phase immune response and is regarded as an endogenous mediator of LPS-induced fever [19]. IL-1 β induces fever and secretion of IL-6 and IL-8, which also plays a role as pro-inflammatory cytokines [2]. IL-1 β is produced mainly by macrophages and plays a significant role in the pathophysiology of endometriosis [20]. Moreover, IL-1 β is important for the initiation and increase of the inflammatory response to microbial infection [21].

NO is synthesized from L-arginine and molecular oxygen by the action of nitric oxide synthase and plays a significant role in host immune defense, vascular regulation, neurotransmission and other systems in normal condition. However, NO in excessive amounts act synergistically with other inflammatory mediators to provoke an inflammatory process. Excessive and uncontrolled production of NO in activated immune cells during inflammation contributes to major destructive forces in tissue injury [1]. COX-2, the inducible COX isoform, has been identified in activated macrophages and constitutes the key enzyme responsible for the high production of inflammatory prostaglandins such as prostaglin E₂, which is also involved in tumor growth and metastasis [22].

In this study, OTEE and EGCG showed anti-inflammatory activity, suppressed TNF- α , IL-6, IL-1 β , COX-2, and NO production. The OTEE and EGCG dose-dependently inhibited TNF- α , IL-6, and NO production. Other studies have also verified that an EGCG ester derivative and theasinensin A in oolong tea exhibited anti-inflammatory activity by reducing the level of pro-inflammatory cytokines and mediators, including inducible nitric oxide synthase, NO, COX-2, IL-12, TNF- α , and monocyte chemoattractant protein (MCP-1) [1,4,8].

OTEE and EGCG have the potential for use as anti-inflammatory drugs, which are shown by their ability to reduce the production of NO, COX-2, IL-6, IL-1 β , and TNF- α in active macrophages. However, oolong tea extract may be more preferable than EGCG because it is far more economical. This research suggests that the anti-inflammatory activity of oolong tea and catechin compounds should be validated in animal models in further studies.

Conflict of interest statement

We declare that we have no conflict of interest.

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