07-Apr-2016

Dear Dr. Widowati,

Your manuscript entitled "Anti-inflammatory Properties of Oolong Tea (Camellia sinensis) Methanol Extract and EGCG (Epigallo Catechin Gallate) in LPS-Induced RAW264.7 Cells" has been successfully submitted online and is presently being given full consideration for publication in Journal of Complementary and Integrative Medicine (DGJCIM).

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Journal of Complementary and Integrative Medicine

Anti-inflammatory Properties of Oolong Tea (*Camellia sinensis*) Ethanol Extract and EGCG (Epigallocatechin Gallate) in LPS-Induced RAW 264.7 Cells

Commented [DJ1]: Seems to be usually spelled as one word.

Abstract

Oolong tea, a partially fermented tea, and the compound (-)-epigallocatechin galate (EGCG), a primary polyphenol in green tea (both made from *Camellia sinensis*), have various biological, such as antioxidant, anti-mutagenic, anticancer, and anti-inflammatory, activities. In the present study, we evaluated the anti-inflammatory activity of oolong tea ethanolic extract (OTEE) and EGCG on lipopolysaccharide (LPS)-induced murine macrophage cell line (RAW 264.7). 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-1β), tumor necrosis factor-α (TNF-α), and cyclooxigenase-2 (COX-2) levels were measured by ELISA, and nitrite oxide (NO) levels measured by a nitrate/nitrite colorimetric assay to determine the inhibition activity of OTEE and EGCG. OTEE had a higher inhibition activity toward NO, COX-2, IL-6, and IL-1β than EGCG; the reverse was seen for TNF-α. However, both OTEE and EGCG suppressed production of NO, COX-2, IL-6, IL-1β, and TNF-α. This research showed that OTEE and EGCG possess anti-inflammatory activity and therefore have potential use as herbal medicines against inflammation. Nonetheless, further study in animal models must be conducted.

Commented [DJ2]: Moved C. sinensis here from original placement after the beverage. Slightly more precise here because "Tea" is a generic term that covers many "hot water extracts" from various plant species.

Commented [DJ3]: This is slightly awkward phrasing, but since these are adjectives modifying "activities" they should be in front of the noun. If placed where you had them, they should be in noun form, since they would be examples of biological activities.

Commented [DJ4]: Should write out acronyms at first mention in Abstract and main body of text. ELISA is probably well-known enough to be abbreviated in Abstract.

Commented [DJ5]: Added "tetrazolium" to give a little more context here.

Commented [DJ6]: Editing to US spelling/conventions throughout.

Introduction

Inflammation is a complex process regulated by pro-inflammatory cytokines and mediators that occur as an innate immune response against 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].

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[12]. Today, many synthetic drugs are used extensively in order to avoid chronic inflammation. However, prolonged consumption of these drugs are sometimes coupled with their own side

Commented [DJ7]: OK to change to "synthetic" here; I assume you'd like to contrast these with natural agents

Commented [DJ8]: Added "their own" since they are themselves used against side effects

effects[13,14]. Naturally derived substances for preventing prolonged inflammation have limited side effects and less 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*) have been shown to have anti-inflammatory activity in suppressing the synthesis and action of many pro-inflammatory mediators. Theasinensins, the primary polyphenols in oolong tea, are thought to potentially inhibit cyclooxygenase-2 (COX-2) expression in lipopolysaccharide (LPS)-activated mouse macrophage-like cells (RAW264.7)[15]. 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 (ROS/RNS)[8,16,17]. 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-1β, TNF-α, COX-2, and NO levels in a LPS-induced murine macrophage cell line (RAW 264.7) model.

Materials and Methods

EGCG and Oolong tea extraction

(-)-Epigallocatechin gallate (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, then extracted with 96% methanol using a maceration technique. The filtrate was filtered and collected every 24 hours until the filtrate became colourless. 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[18].

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% CO_2 in humidified atmosphere until confluent (80–90%). Trypsin-EDTA (Biowest) was used to harvest the cells which then seeded on plates for the assays[19,20].

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 (MTS) assay (Promega, Madison, WI, USA). A total of 100 µl of medium (DMEM supplemented with 10% FBS, and 1% antibiotic-antimycotic) containing around 5 x 10^3 RAW 264.7 cells was seeded in each well of a 96-well plate, which was then incubated for 24 hours at 37°C, 5% CO_2 in a humidified atmosphere. The medium was washed from the cells and the cells were then supplemented with 90 µl of fresh medium and 10 µl of OTEE (100, 50, and 10 µg/ml) or EGCG (100, 50, and 10 µM), and incubated for 24 hours. To all of the wells, 20 µl of MTS was added and the plate was incubated at 37 °C, 5% CO_2 for 3 hours. The absorbance was measured at 490 nm. Cells without treatment served as a control, and % viability was obtained from the difference in viable cells from each treatment from the control[18–21].

LPS-induced RAW 264.7

A 6-well plate was used for growing RAW 264.7 cells. To induce inflammation, 1 µg/ml lipopolysaccharide (LPS from *E. coli*) (Sigma) was added into each well of the positive control (LPS-induced RAW 264.7 without OTEE and EGCG), negative control (RAW 264.7 only), and treatment wells, to which

Commented [DJ9]: Okay spp. Placement here because fairly clear tea plant is being discussed, not beverage.

Commented [DJ10]: "possess the potential" is a bit vague. "are thought to" (or something like that) implies a stronger suspicion or hypothesis.

Commented [DJ11]: Ok to add for context here?

Commented [DJ12]: I think I would give a little information about the tea here (for readers not familiar with it) Maybe "The partially broken, fermented tea leaves of oolong tea…" Or was it whole leaf?...Or maybe even one or two sentences about fermentation process, fermentation organisms involved….

Commented [DJ13]: "filtrate was filtered" is a little unclear here (seems circular). You probably need another sentence just before this describing the maceration and suspension separation technique in a little more detail. What is being filtered in this first step and how? Or do you just mean just the methanol solvent was collected, filtered and replaced every 24 hrs?

Commented [DJ14]: Assume "pellet" is meant here; maybe powder? Often the color of the pellet seems to be mentioned at this point (I'm not sure why…tradition?)

Commented [DJ15]: Wasn't methanol used as extraction solvent? Would "alcohol-extracted" be more accurate here and throughout?

Commented [DJ16]: Did you combine the pellet fractions before storage? As phrased currently, it implies you did not.

Commented [DJ17]: Appropriate full name for FBS here?

Commented [DJ18]: "A total of" is a "filler" phrase to avoid starting sentence with number.

Commented [DJ19]: Absorbence of just a sample of the media or the cell wells themselves (cells plus media)? (Apologies if this is common knowledge in your field)

Commented [DJ20]: A little unclear here; does this rephrasing preserve your intended meaning?

Commented [DJ21]: It's good to note the purpose of a step, unless obvious (esp. here, the stress against which you are testing treatments; also avoids starting sentence with number :)

OTEE (50 and 10 µg/ml) or EGCG (50 and 10 µM) were previously added. The plate was then incubated for 24 hours. Content in each well was then centrifuged, and the cell-free supernatant was used for the IL-6, IL-1β, COX2, NO, and TNF-α assays[19,20].

Commented [DJ22]: At what speed?

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, MultiSkan 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[19,20].

COX-2 level assay

Quantification of COX-2 used a Mouse PTGS2/COX-2 ELISA kit protocol (Elabscience). 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.

TNF-α level assay

Quantification of TNF-α used a Mouse TNF-α ELISA MAX™ Standard kit protocol (BioLegend). 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[19,20].

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[19,20].

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[19,20].

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 mean ± standard deviation of 3 independent experiments.

Commented [DJ23]: Present tense because you are referring to the current paper.

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 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

Commented [DJ24]: Values properly sorted here?

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, 30.95% and 56.69%, respectively (Tables 2, 5), while 50 µM of EGCG provided the highest TNF- α inhibition activity (44.37%) (Table 4). Lastly, 10 µg/ml of OTEE showed the highest COX-2 and IL-1β inhibition activity by 47.46% and 28.49%, respectively (Table 3, and 6).

Commented [DJ25]: Modified slightly here to avoid starting sentence with number.

Commented [DJ26]: Changed to "lastly" ("finally", etc. OK too). "moreover" implies presentation of more evidence to support an argument; here I think you are just highlighting results.

Discussion

As noted above, several studies have reported that oolong tea and tea polyphenols exerted biological, including anti-oxidant, anti-mutagenic, anticancer, and anti-inflammatory, effects. In this study, we evaluated the anti-inflammatory properties of OTEE and EGCG toward inhibition of TNF-α, IL-6, IL-1β, COX-2, and NO production on 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 (TSA), a major polyphenol in oolong tea, could suppress the expression of inflammatory mediators such as COX-2 and prostaglin E2 (PGE2) by attenuating cellular signaling, including the mitogen-activated protein kinase (MAPK) and NF-kB pathways[22]. Nagai et al. (2002), 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 mM, and also demonstrated that EGCG could protect against ischemic neuronal damage by deoxidizing peroxynitrate/peroxynitrite, which is converted to a NO or hydroxyl radical[8]. Moreover, EGCG has been shown to suppress NO production by inhibiting inducible nitric oxide synthase (iNOS) expression in LPS/cytokine-induced human chondrocytes and in LPS/cytokine-induced murine macrophages by blocking NF-kB activation[23,24].

Commented [DJ27]: Added this here, otherwise should cite this sentence.

Commented [DJ28]: Ok change here?

Commented [DJ29]: Usually not italicized.

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[25]. In macrophages or monocytes, LPS induces inflammatory response by initiating signal transduction through toll-like receptor 4 (TLR-4) to activate expression of pro-inflammatory cytokines and mediators, including NO, IL-1, IL-6, and TNF-α[22,26,27]. As seen in the positive control, LPS-induced RAW 264.7 resulted in a significant increase of TNF-α, IL-6, IL-1β, 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 µg/ml) and EGCG (50 and 10 µM) 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-κB, and stimulating NO production[5,7,28,29]. Because of its multiple roles in inflammation, TNF-α has been targeted for screening as an anti-inflammatory agent[30]. IL-6 and IL-1β are synthesized mainly by macrophages and have their own activities and effects in inflammation. IL-6 activates neutrophils and NK-cells[6], plays a role in the acute-phase immune response and is regarded as an endogenous mediator of LPS-induced fever[31]. IL-1β induces fever and secretion of IL-6 and IL-8, which also play a role as pro-inflammatory cytokines[4,6]. IL-1β is produced mainly by macrophages and plays a significant role in the pathophysiology of endometriosis[32]. Moreover, IL-1β is important for the initiation and increase of the inflammatory response to microbial infection[33].

Commented [IS30]: Added "multiple" here to emphasize this large scope of actions.

Commented [IS31]: To double-check: wouldn't this be "inhibitors of TNF-alpha have been targeted" ?

NO is synthesized from L-arginine and molecular oxygen by the action of nitric oxide synthase (NOS). NO plays a significant role in host immune defense, vascular regulation, neurotransmission and

other systems in normal condition. However, NO in excessive amounts acts 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]. NO increases the expression of COX-2 via modulation of NF-kB[34]. 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 (PGs) such as PGE_2, which is also involved in tumour growth and metastasis[35]. Due to the rapid half-life of NO *in vivo,* the quantity of nitrite accumulated in culture media was measured as an indicator of NO production[12].

In this study, OTEE and EGCG showed anti-inflammatory activity. OTEE and EGCG suppressed TNF-α, IL-6, IL-1β, 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 TSA in oolong tea exhibited anti-inflammatory activity by reducing the level of pro-inflammatory cytokines and mediators, including iNOS, NO, COX-2, IL-12, TNF-α, and monocyte chemotactic protein (MCP-1)[1,8,15,17].

Conclusion

Oolong tea ethanol extract 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-1β, 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.

Ref: APJTB_2016_899
Title: Anti-inflammatory Properties of Oolong Tea (Camellia sinensis) Ethanol Extract and EGCG (Epigallocatechin Gallate) in LPS-Induced RAW 264.7 Cells
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2. Please provide detailed department or subuint information for affiliations 1,3,4.

3. The discription of the grouping of the treatment in 2.4. is very unclear. Please rephrase this part for clarity.

4. Please provide Foundation Name and Grant No. (Don't forget to provide the Grant No.).

5. Update the references and make sure half of the references are in recent 3 years!!!!! (2012-2015), another one-fourth references are in recent 5 years (i.e. 2010-2015). Old references are better replaced with updated ones. References must meet these requirements!!!! Citations should be labeled with square bracket and should appear in front of the period or comma at the end of the sentence/clause. All references should be cited. It is better to include citation from Pubmed, the more the better.

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