ASIAN JOURNAL OF PHARMACEUTICAL AND CLINICAL RESEARCH

INNOVARE ACADEMIC SCIENCES
Knowledge to Innovation

Vol 12, Issue 2, 2019

Online - 2455-3891
Print - 0974-2441

Research Article

INTERLEUKIN-1B AND CYCLOOXYGENASE-2 PROINFLAMMATION ANALYSIS AND *IN SILICO* DOCKING NUCLEAR FACTOR KAPPA B ON ENDOMETRIOSIS CELL CULTURE GIVEN HEPTYL GALLATE AND OCTYL GALLATE TREATMENT

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Received: 24 October 2018, Revised and Accepted: 11 January 2019

ABSTRACT

Objective: The aim of this study is to analyze the effect of octyl gallate and heptyl gallate toward the regulation of interleukin-1β and cyclooxygenase (COX)-2 proinflammatory factor on endometriosis cell culture and analyze its activity toward nuclear factor kappa B (NFkB) target protein through *in silico* docking technique.

Methods: *In vitro* study was performed on endometriosis cells cultured treated with two dosages each of heptyl and octyl gallate (51.2 μg/mL and 102.4 μg/mL) for 48 h, then followed by 10 ng/mL lipopolysaccharides (LPS) induction for 24 h. The positive control group was treated by LPS induced and the negative control was treated without LPS. Inflammation regulation was evaluated with enzyme-linked immunosorbent assay technique and *in silico* docking analyzed using bioinformatics technique.

Results: Molecular docking analysis with gallic acid and their derivatives showed that more stable affinity and stronger binding found on octyl gallate than heptyl gallate and gallic acid at the active site of NFkB.

Conclusions: Based on this study results, octyl gallate and heptyl gallate were proven to be able to reduce COX-2 proinflammatory factor through NFkB pathway as an inflammatory regulator; thus, it has the potential to be developed as a therapy for endometriosis.

Keywords: Heptyl gallate, Octyl gallate, Endometriosis, *In silico* docking nuclear factor kappa B, Cyclooxygenase-2, Interleukin-1β.

 DOI: http://dx.doi.org/10.22159/ajpcr.2019.v12i2.30399

INTRODUCTION

Endometriosis is a pathological disease caused by the uncontrolled proliferation of ectopic tissue outside the endometrial cavity [1]. Giudice (2010) defines endometriosis as an inflammatory condition characterized by tissue lesions such as endometrium outside the uterus and generally associated by pelvic pain and infertility [2]. The endometriosis lesions growth could trigger the concentration of proinflammatory cytokines in the peritoneal cavity leading to chronic inflammation [3]. Proinflammatory factors such as interleukin (IL)-1β and tumor necrosis factor (TNF) -α activate the nuclear factor-kappa B (NF-kB) signaling pathway and hypoxia-inducible factor -1α, thus increasing the expression of cyclooxygenase (COX)-2 in endometriosis. NF-kB is suggested in mediating the occurrence of inflammation thereby increasing the secretion of several cytokines, including TNF-α, IL 1, IL 6, IL8, IL 10, interferon gamma, macrophage inflammatory proteins 1-2, RANTES, intercellular adhesion molecule-1, matrix metallopeptidase-9, and COX-2 which also influence the proliferation excess, invasion, angiogenesis, and persistence of ectopic endometriosis growth [4-6].

IL-1β is a proinflammatory cytokine that stimulates endometriosis cells producing cytokines and growth factors which have a role in adhesion, growth, invasion, inflammation, and angiogenesis in endometriosis [7,8]. Previous studies showed that IL-1β induced the expression of vascular endothelial growth factor (VEGF) and COX-2, angiogenic factors, in some cancers. As already known, COX-2 and VEGF play an important role in the angiogenesis process in endometriosis [9,10].

At present, the management of endometriosis focused on hormonal therapy and conservative surgery, resulting in approximately 50% of women with endometriosis reduced pain [11], and the recurrence rate after endometriosis is around 11–32% within 1–5 years [12]. Therefore, it is suggested to develop an advanced and effective method as promising management of endometriosis.

Derivatives of alkyl ester from gallic acids such as heptyl gallate and octyl gallate have the ability to suppress the proliferation and induce apoptosis in some cancer cells [12]. The novelty of this study was to analyze the effects of alkyl gallate derivatives, namely heptyl gallate and octyl gallate as anti-inflammatory candidates in endometriosis cells. The effectiveness of gallic acid is influenced by the ability of entering into the cell and affecting the biological activity of the cell. Gallic acid has hydrophobic properties, influenced by the length of the alkyl group carbon chain, which supports the penetration into the cell. The difference in gallic acid and alkyl ester gallate derivatives lies in the number of carbon atoms bound to the side chain, thus giving physicochemical characteristics, especially lipophilicity, which can be seen from the partition coefficient (C log P) [13]. Heptyl gallate and octyl gallate are modifications of gallic acid by adding carbon chains to the alkyl group of the tool so that it is more hydrophobic. The addition of OH groups to the groups of gallic acid derivatives (heptyl gallate and octyl gallate) increases the solubility and hydrophobicity of the substance so as to facilitate the penetration and increase of the biological activity of the natural substances within the cell. Lipophilic chain length on the side chain alkyl ester form affects the affinity and cell membrane permeability to these substances [14,15]. Our previous research proved that octyl gallate suppressed the expression of nuclear factor-kappa B (NF-kB) mRNA, proinflammatory pathway transcription factor, and the proliferation of endometriosis cell *in vitro* [16]. Therefore, it is necessary to prove the mechanism through bioinformatics analysis

with *in silico* docking techniques to obtain compounds which more potential, stable, and has specific activities in inhibiting NFkB [17,18].

The purpose of this study was to identify the bond strength and potential inhibition of gallic acid derivatives, heptyl gallate, and octyl gallate, toward the protein target, NF-κB through *in silico* docking technique and the effect toward the regulation of proinflammatory cytokines, IL-1β and COX-2, in primary cultures of endometriosis cells.

MATERIALS AND METHODS

Materials

Gallic acid was synthesized by the Chemical Department FKUI, fetal bovine serum (FBS), Fungizone, powder Dulbecco's modified eagle's medium F-12 (DMEM F-12) from Gibco/Life Tech USA, penicillin/streptomycin (Sigma-Aldrich), phosphate buffered saline (Merck. IL1β), and COX-2 ELISA Kit (Quantikine R and D and MyBioSource).

In silico docking analysis

In silico docking, the study was performed to analyze docking energy values (ΔG) and amino acids association in the process of interaction between macro NFkB molecules and ligands (octyl gallate and heptyl gallate) using software Marvin Sketch, AutoDock, PyMOL, and LigPlus which are designed for docking.

Isolation and primary culture of endometriosis tissue

The endometriosis tissues patients' were obtained using laparoscopy procedure, they were put in the transport medium (DMEM F-12 containing 2% penicillin/streptomycin and 2% Fungizone). Then endometriosis cells are obtained by isolating enzymatically cells using Type IV collagenase and culturing it until the cells reached 6×10^6 in complete medium (DMEM F-12 containing 1% penicillin/streptomycin, 1% Fungizone, and 20% FBS). The 2.5×10^4 cells/well were grown in 12 well plates, then treated with heptyl and octyl gallate with two doses (51.2 μg/mL and 102.4 μg/mL) for 48 h, followed by induction of 10 ng/mL lipopolysaccharides (LPS) for 24 h. The positive control group only induced by LPS, and negative control was treated without LPS.

Analysis of levels of cytokines IL-1β and COX-2

Inflammatory regulation was assessed from the level of cytokines IL-1β and COX-2 with ELISA (Quantikine, R and D system, MyBioSource) techniques. Analysis of level IL-1β performed with add 200 μL of standard, sample, and control to each well, then incubated for 2 h at lid temperature with adhesive cover strips. After 1 h, repeat the steps for washing, followed by adding 200 μL of substrate solution to each well, cover with aluminum foil and incubate for 20 min at room temperature. Then, add 50 μL of stop solution to each well, then the color changes from blue to yellow, then read the OD of each well with a spectrophotometer within 30 min at a wavelength of 450 nm and correction at 540 or 570 nm. Analysis of level COX-2 performed with add 100 μl standard, sample, and control to each well, then incubated for 90 min at 37°C cover with adhesive cover strips, biotinylated COX-2 antibodies prepared 30 min before incubation is complete, after incubation do aspiration and washing by adding 200 μL wash buffer, repeat up to 3 times washing. At the end of each wash, dry it by placing the inverted plate on the tissue. Put 100 μL of biotinylated COX-2 antibody on each well, cover it with the adhesive cover strip by incubating for 60 min at 37°C, before incubation is finished 30 min before preparing enzyme-conjugate. After incubation, complete aspiration and washing by adding 200 μL of wash buffer, repeating up to 4 times of washing. At the end of each wash, dry it by placing the inverted plate on the tissue. Then, add 100 μL of enzyme-conjugate to each well, cover with the adhesive cover strip and incubate for 30 min at 37°C, re-aspirate and wash with 200 μL of wash buffer, repeat up to 6 times of washing. At the end of each wash, dry it by placing the inverted plate on the tissue, then add 100 μL of Color Reagent A to each well, incubate at 37°C until the color turns dark in 30 min, add 100 μL of Color Reagent C to each well, step finally, read the OD of each well with a spectrophotometer within 10 min at a wavelength of 450 nm and correction at 540 or 570 nm.

Table 1: Results of *in silico* docking between ligands and NFkB

No	Compound	Binding energy score (kkal/mol)	pKi (μM)	HB
1	Gallic acid	−7.66	2.42	5
2	Heptyl gallate	−7.68	2.37	2
3	Oktyl gallate	−7.98	1.41	3

NFkB: Nuclear factor kappa B, pKi: Inhibition constant, HB: Hydrogen bonds

RESULTS

Based on the results of *in silico* docking analysis, the docking energy (binding energy score/ΔG) of gallic acid compounds, heptyl gallate and octyl gallate as ligand against protein NFkB targets respectively −7.66 kkal/mol, −7.68 kkal/mol and −7.98 kkal/mol. ΔG shows the strength of the ligand affinity with the target protein, in which the higher of the negative ΔG value the interaction and conformation between ligand and protein will be more constant and stable [19]. In this study, the octyl gallate showed a stronger and more stable affinity toward NFKB than heptyl gallate and gallic acid.

The amino acids through hydrogen bonds (HB), Tyr285, Lys221, Ser222, Ser226, Ser220, Pro223, and Lys252, performed the bond between ligands with NFkB residues at a distance of <3,31 Å (Fig. 1). HB which could increase the ligand activity was found in the amino acid residues Ala225. The octyl gallate had 3 HB with amino acids Lys221, Ser2260, and Ala225, while heptyl had two amino acids, Ser226 and Lys283. The quantity of HB made ligand interactions between NFkB proteins and compounds heptyl gallate and octyl gallate became stronger. Thus, the octyl gallate showed more potent for interaction and inhibitory activity toward NFKB than the heptyl gallate. Another docking indicator is the value of the inhibition constant (pKi), which showed the inhibitor value between the ligand-protein complexes. A low pKi value is a good indicator for the formation of a ligand-protein complex. The result of ΔG, pKi, and HB is presented in Table 1.

The effect of heptyl gallate and octyl gallate on primary endometriosis cells toward pro-inflammatory cytokines IL-1β in this study showed

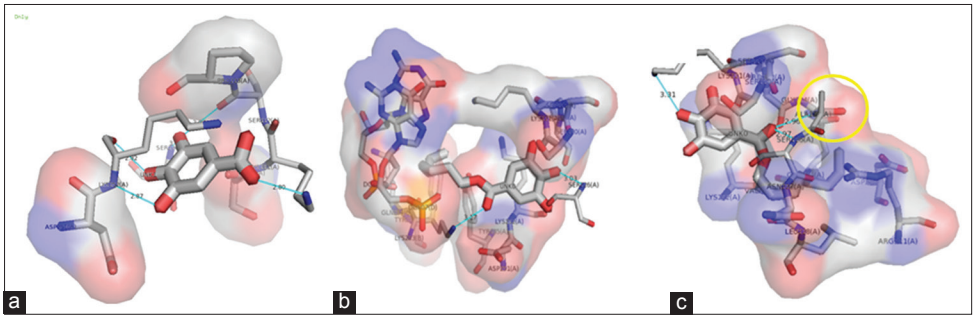

Fig. 1: Visualization of nuclear factor kappa residual interactions with compounds (a) Gallic acid, (b) Heptyl gallate, (c) Octyl gallate

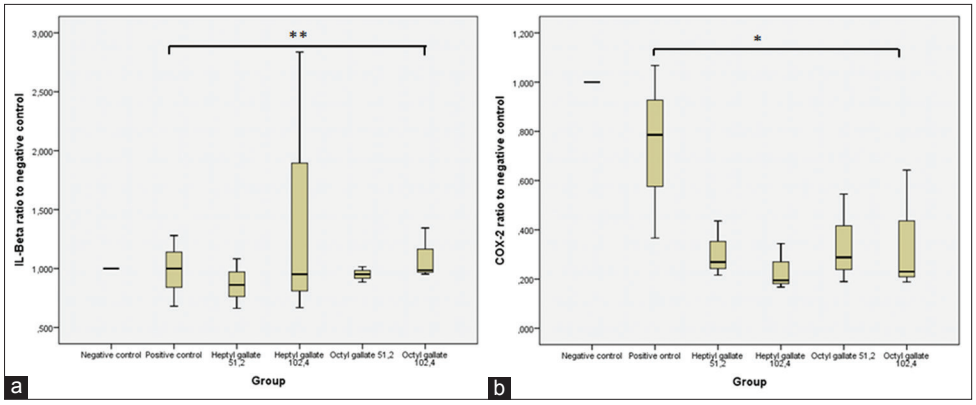

Fig. 2: The production ratio of (a) interleukin-1β level **p>0.05=no significant difference compared to the group in endometriosis cells given heptyl gallate and octyl gallate compared with positive controls and (b) cyclooxygenase-2level *p<0.05=significantly different compared to endometriosis cells given heptyl gallate and octyl gallate compared with positive controls. Describe HG: Heptyl gallate, OG: Octyl gallate, Dosage: 51.2 µg/mL and 102.4 µg/mL. n=3

that both compounds had the potential to suppress IL-1β generation, but there was no significant difference between the two compounds (p>0.05). On the other hand, there were significant differences in inhibition of COX-2 production in both heptyl and octyl gallate (p=0.03), although the difference in dosage was not affect the strength of the production effect (Fig. 2).

DISCUSSION

In silico docking analysis between gallic acid, heptyl gallate, and octyl gallate as ligands with NFkB target proteins regarding the affinity, showed that octyl gallate was stronger and more stable in affinity compared to heptyl gallate and gallic acids, thus octyl gallate was suggested more potential in inhibiting the NFκB pathway than heptyl gallate and gallate acid. This was in line with the results of our previous study that octyl gallate more suppressed the relative expression of NFκB mRNA in endometriosis cells using quantitative real-time polymerase chain reaction identification compared to heptyl gallate and gallic acid [16,18,20]. The results of the study using *in silico* docking of gallic acid derivative compounds on the dihydrofolate reductase malarial receptor showed that octyl gallate had strong interactions and had the greatest inhibitory activity [21].

The suppression of NFκB expression is caused by several factors, one of which is suppressed by proinflammatory cytokines IL-1β. Although in this study IL-1β production was not statistically significantly decreased, both heptyl and octyl gallate had the potential to suppress NFkB expression. Another possibility that causes NFκB inhibition is still poorly understood, and further research is needed.

Studies on the treatment of DLBS2411 can increase IKKα phosphorylation and activate NF-kB, where high levels of NFkB can increase the expression of COX-2 and prostaglandin E2 [22]. The remarkable thing from this study was that the inhibited effect on NFkB mRNA expression also suppressed the COX-2 production. Previous research reported that COX-2 and VEGF had an important role in angiogenesis in endometriosis [9,10] and high COX-2 production also played a role in increasing pain in patients [23]. With the ability of octyl gallate and heptyl gallate which can affect the mechanism of proinflammatory regulation by suppressing the NFkB pathway and COX-2 secretion, the inflammatory process could be prevented and or suppressed, so the process of proliferation, invasion, angiogenesis, and persistence of ectopic implants in endometriosis cells was not occurred. This was in line with the results of our previous studies that demonstrated the ability of gallate acid, heptyl gallate, and octyl gallate to reduce cell proliferation and increase apoptotic endometriosis cells [24,25]. Likewise, previous studies reported that the alkyl ester derivatives of gallic acid such as heptyl gallate and octyl gallate had the ability to suppress proliferation and induced apoptosis in some cancer cells [12].

CONCLUSIONS

In the *silico* docking study showed octyl gallate had a stronger binding and more stable affinity in inhibiting NFkB protein because it had the highest docking energy (ΔG), the lowest pKi, and the highest number of HB compared to heptyl gallate and gallic acid. In addition, we proved that octyl gallate and heptyl gallate have the potential to suppress IL-1β secretion driving to NFκB mRNA expression and COX-2 decrease in endometriosis cells. Octyl gallate and heptyl gallate can be developed as promising agents in the management of endometriosis through their inhibitory effects in the proinflammatory pathway. *In vivo* study is still needed to prove the effects of these two compounds as endometriosis management.

ACKNOWLEDGMENTS

The funding of this research was supported by PUPT 2017 grant. The authors are thankful to Dr. R. Muharam, SpOG (K) for providing the necessary samples for this research and also to Dr. Ade Arsianti for preparing octyl gallate and heptyl gallate.

AUTHORS' CONTRIBUTIONS

Dr. Arleni has the role of supervising research and directing the making of manuscripts; Mrs. Fajar Sulistya Utami performed the experiment and wrote the manuscript; Dr. Heri helped in supervising data processing analysis, and Mrs. Rahmi Budiarti performed endometriosis cells isolation and culture.

CONFLICTS OF INTEREST

We declare there are no conflicts of interest in this research.

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