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Original Article

# *IN VITRO* ANTI-NEUROINFLAMMATORY EFFECT OF GENISTEIN (4',5,7-TRIHYDROXYISOFLAVONE) ON MICROGLIA HMC3 CELL LINE, AND *IN SILICO* EVALUATION OF ITS INTERACTION WITH ESTROGEN RECEPTOR-β

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**ABSTRACT**

**Objective:** This study was aimed to evaluate the role of genistein or 4',5,7-trihydroxyisoflavone as a phytoestrogen in the treatment of estrogen deficiency-induced neuroinflammatory. The specific objectives of this study were to determine the anti-neuroinflammatory effect of genistein through measurement of MHC II and Arg1 expressions on microglia HMC3 cell line, as well as to prove that the effect occurs in an ER-dependent manner, through the measurement of free-ERβ expression.

**Methods**: The cells were cultured in 24-well microplates, induced with 10 ng IFN-γ, and incubated for 24 h to activate the cell to $M_1$ phenotype which has pro-inflammatory characteristics. Genistein with a concentration of 50 μM was added to the cells. The expression of MHC II, Arg1, and free-ERβ as markers was tested through an immunocytochemistry method and measured using the CLSM instrument. *In silico* approach was also conducted to determine the interaction between genistein and ERβ, compared to 17β-estradiol. Genistein structure was prepared with Avogadro 1.0.1, and molecular docking was done using PyRx 0.8 software. Biovia Discovery Studio Visualizer 2016 was used to visualize the structure of genistein against 3OLS protein. The physicochemical characteristics of genistein were analyzed using the SwissADME web tool.

**Results**: Genistein can decrease MHC II expression and increase Arg1 expression in microglia HMC3 cells compared to negative controls ($p<0.005$), with expression value of 472.577±26.701 AU and 114.299±6.578 AU. But, genistein cannot decrease the free-ERβ expression in cells ($p<0.005$). The results of *in silico* analysis showed that genistein is an ERβ agonist.

**Conclusion**: Genistein shows anti-neuroinflammatory effects by decreasing the MHC II expression and increasing Arg1 expression in the microglia HMC3 cell line. However, this effect does not occur through the binding of genistein to ERβ, but it is likely to occur through the binding of genistein with other types of ER.

**Keywords:** Genistein, Anti-neuroinflammatory, Microglia HMC3 cell line, Phytoestrogens


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## INTRODUCTION

The global prevalence of neurodegenerative diseases has been increasing in recent decades [1]. One of the main causes of neurodegenerative diseases is neuroinflammatory. For postmenopausal women, neuroinflammatory is triggered by estrogen deficiency. In the other words, estrogen deficiency triggers postmenopausal women to be prone to neuroinflammatory, which can causes some neurodegenerative disorders [2-4]. In neuroinflammatory, there is an increase of the number of microglia cells that are activated in the $M_1$ polarity state which has pro-inflammatory characteristics due to estrogen deficiency. The increasing of activated microglia cells then causes an increase in inflammatory cytokines, such as interleukin-1 (IL-1), IL-6, tumor necrosis factor-α (TNF-α), and nitric oxide (NO), as well as major histocompatibility complex II (MHC II) [5, 6]. Continuous M1 polarity state activation of microglia cells can cause prolonged inflammation and induces hippocampal neuron cell death leading to decreased cognitive function [7-9].

Inhibition of neuroinflammatory progression can be conducted to prevent neurodegenerative diseases. The logic method is to provide estrogen replacement compounds to activate microglia cells towards the $M_2$ polarity state, which has anti-inflammatory characteristics. Activation of microglia cells in $M_1$ polarity causes pro-inflammatory conditions that are characterized by an increase of inflammatory cytokines. In contrast, activation of microglia cells in $M_2$ polarity causes an anti-inflammatory condition characterized by decrease of inflammatory cytokines, as well as an increase in the expression of IL-10, IL-13, T-cell growth factor-β (TGF-β), and arginase 1 (Arg1) [7-9].

Phytoestrogens are compounds derived from plants which have similar structure to estrogen. Biologically, they can replace the function of estrogen in maintaining homeostasis in the brain, both in conjunction with estrogen receptors (ER-dependent) or not in conjunction with the receptors (ER-independent) [10]. Thus, using phytoestrogens can be an alternative treatment for estrogen deficiency-induced neuroinflammatory [11]. Genistein or 4',5,7-trihydroxyisoflavone with molecular formula $C_{15}H_{10}O_5$ (fig. 1) is an example of phytoestrogens. Genistein is a plant naturally occurring compound belonging to the isoflavone group. This compound can be found in various species of Leguminoceae or Fabaceae [12]. Genistein was known to be able to bind estrogen receptors (ER) and produce many estrogenic effects [13-16], thus, it is potential to be used as an agent of neuroinflammatory therapy.

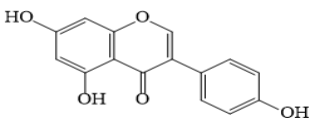


**Fig. 1: Molecular structure of genistein**

This research aimed to determine the anti-neuroinflammatory effect of genistein through measurement of MHC II and Arg1 expression on human microglia clone 3 (HMC3) cell line (ATCC® CRL-3304™) [17] in an *in vitro* testing, as well as to know that the effect occurs in an

ER-dependent manner, through the measurement of free-ERβ expression. The estrogen deficiency condition was created by induction of interferon-γ (IFN-γ) to the microglia HMC3 cells culture, and the measurement of markers was done using immunocytochemistry method and confocal laser scanning microscope (CLSM) instrument.

In addition to *in vitro* testing, we also confirmed the interaction pattern between genistein and the estrogen receptor *in silico*, by conducting molecular docking between genistein and 3OLS protein-containing histidine, glutamine and arginine on the active site of this protein. Research on genistein as a neuroprotective agent has been done quite a lot, but the effect of genistein as a neuroprotective through an anti-neuroinflammatory mechanism in HMC3 microglia cells has never been done before.

## MATERIALS AND METHODS

### Materials

The microglia HMC cell line was purchased from American Type Culture Collection (ATCC® CRL-3304™) Manassas, USA [17]. Genistein, eagle's minimum essential medium (EMEM), dimethyl sulfoxide (DMSO), paraformaldehyde (PFA), fetal bovine serum (FBS), phosphate buffer saline (PBS), serum bovine albumin (BSA), Tween 80, IFN-γ, Triton X100, anti-rabbit secondary antibody fluorescein isothiocyanate (FITC), anti-mouse secondary antibody rhodamine, were purchased from Sigma-Aldrich, Missouri, USA; penicillin, streptomycin, anti-rabbit MHC II primary antibody (ab114224), anti-rabbit ERβ primary antibody (ab3576), were purchased from Abcam, Boston, USA; anti-mouse Arg1 primary antibody (sc-166920) was purchased from Santa Cruz Biotechnology, California, USA.

### Cell culture

Preparation of cell culture was conducted according to a procedure developed by Central Laboratory of Life Sciences, Universitas Brawijaya, Indonesia [18-20]. Microglia HMC3 cell line was cultured in a 25 $cm^2$ flask containing±250.000 cells with a complete medium containing 5 ml EMEM, 10% FBS, and 1% penicillin-streptomycin. The cells culture was then incubated in a 5% CO2 incubator at 37 °C for 6 d, until the cells growth reached 80% confluent in the flask.

### Genistein solution

A 50 µM genistein sample solution was prepared by mix 40 µl of 1 mmol genistein solution with 0.8 ml complete medium containing 5 ml EMEM, 10% FBS, and 1% penicillin-streptomycin.

### Measurement of MHC II, Arg1, and free-ERβ

The effects of genistein on microglia HMC3 cell lines were measured according to a procedure developed by Central Laboratory of Life Sciences, Universitas Brawijaya, Indonesia [19-20]. After cultured cells reach 80% confluence, then the induction of IFN-γ was performed. 10 ng IFN-γ was added into the cells, and the culture was incubated for 24 h. The cultured cells were then rinsed with PBS and treated with 50 µM genistein for 48 h. The cells then rinsed again with PBS and fixed with 4% PFA, Triton X100, BSA, and anti-rabbit MHC II primary antibody for single staining methods. After the previous procedure, a double staining method was performed in different 24-well microplates with anti-mouse Arg1 primary antibody and anti-rabbit ERβ primary antibody. The cells were then incubated at 4 °C overnight. As the final step of the procedures, an anti-rabbit secondary antibody FITC and anti-mouse secondary antibody rhodamine were added before the cells were analyzed using a CLSM instrument at 488 nm and 543 nm.

### *In silico* evaluation

The three-dimensional structure of estrogen receptor (ERβ) was obtained from Protein Data Bank (http://www.rcsb.org) with code 3OLS [21]. Initial preparation was done to separate the natural ligand of ERβ–that is 17β-estradiol-from the protein using Biovia Discovery Studio Visualizer 2016. The natural ligand and genistein molecular structures were prepared with Avogadro 1.90.0 for energy optimization by using the MMF94s method. Molecular docking and simulation of the docking were conducted using PyRx 0.8 software [22, 23]. The complex of receptor-ligand obtained from docking simulation was visualized using Biovia Discovery Studio Visualizer 2016. Further analysis was done using the SwissADME web tool to predict the physicochemical properties of genistein and the natural ligand.

## RESULTS

Visualization of marker expression in microglia HMC3 cells can be seen in fig. 2 and fig. 3, while the results of the analysis of marker expression are as shown in table 1. In fig. 2 and fig. 3, the treatment of genistein decreased MHC II expression significantly compared to negative control with p=0.002. The decrease in MHC II expression was indicated by the decrease in the fluorescence intensity of the microglia HMC3 cell line. The induction of genistein increases Arg1 expression significantly compared to negative control with p<0.001, as well as increases the free-ERβ expression significantly compared to negative control with p<0.001. The increase in Arg1 expression was indicated by an increase in the red color intensity of microglia HMC3 cell line, while the increase in free-ERβ expression was indicated by an increase in the green color intensity of the microglia HMC3 cell line.

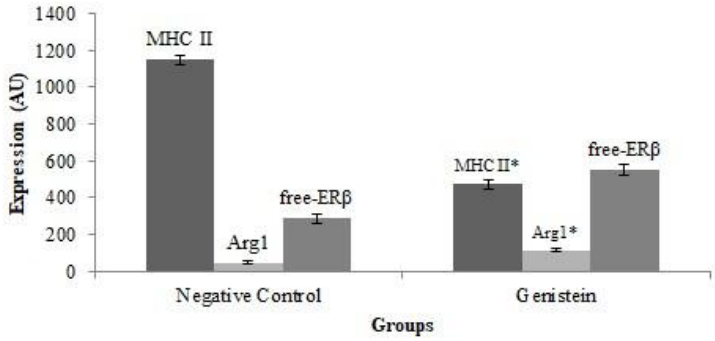


**Fig. 2: The expression of MHC II, Arg1 and Free-ERβ after genistein induction in microglia HMC3 cell line. Each value is expressed as mean±SD. Significant differences compared to a negative control (*) at p<0.05**

**Table 1: The expression value of MHC II, Arg1, and free-ERβ in arbitrary unit (AU) after genistein induction in microglia HMC3 cell line**

| Groups | MHC II (AU) | Arg1 (AU) | Free-ERβ (AU) |
|---|---|---|---|
| Negative control | 1149.399±25.810 | 47.080±6.799 | 287.735±26.107 |
| Genistein | 472.577±26.701* | 114.299±6.578* | 553.295±29.694* |

Note: Each value is expressed as mean±SD. Significant differences compared to negative control (*) at p<0.05.

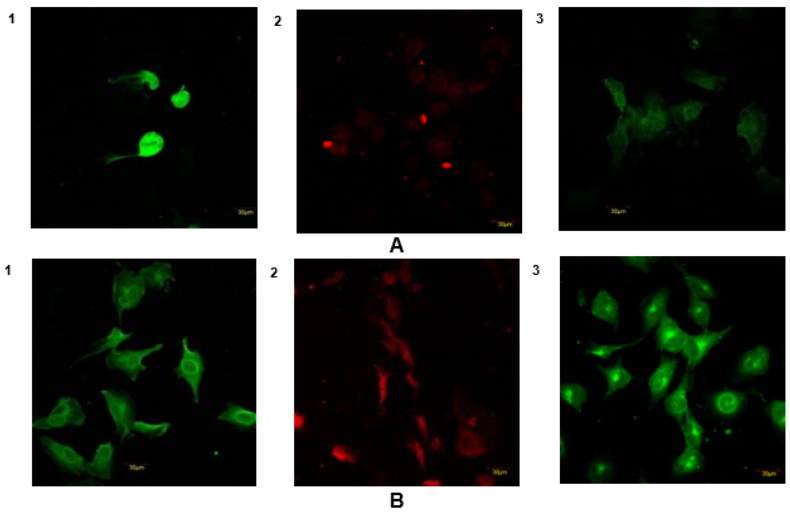


**Fig. 3: The immunofluorescence visualization of markers in microglia HMC3 cell lines. (A) Negative control; (B) Genistein: (1) MHC II expression, (2) Arg1 expression, (3) Free-ERβ expression**

The results of *in silico* evaluation on the interaction of both genistein and natural ligand (17β-estradiol) with the ERβ can be seen in table 2. A comparison of pharmacophore distance (bond distance) and amino acid bonds between 3OLS protein and 17β-estradiol and genistein is as shown in fig. 4. Compounds or ligands are categorized as agonists of the 17β-estradiol if they have a bond distance and pharmacophore groups similar to 17β-estradiol. Besides that, they should bind the same amino acids in the 3OLS protein as 17β-estradiol. That is, if it has a bond distance of about 10.862 Å, and has one pharmacophore group that binds His 475, and another pharmacophore group binds Glu 305 or Arg 346. These results indicate that genistein is an ERβ agonist like 17β-estradiol; thus, it has a high potential to be used as a drug in neuroinflammatory therapy.

**Table 2: Docking parameters of the interactions of genistein and 17β-estradiol with 3OLS protein**

| Ligand | Binding affinity (kcal/mol) | Rmsd average (Å) | Amino acid and bond type | Pharma-cophore distance (Å) | TPSA ≤ 140 | Lipinski's rule of five | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | BM (g/mol) | Log P | H-bond acceptors | H-bond donors |
| Genistein | -8.6 | 0.000 | His475 (Hydrogen)<br>Glu305 (Hydrogen)<br>Arg 346 (Unfavorable) | 12.136 | 90.90 | 270.24 | 0.52 | 5 | 3 |
| 17β-estradiol | -10.5 | 0.000 | His475 (Hydrogen)<br>Glu305 (Hydrogen)<br>Arg 346 (Hydrogen) | 10.862 | 40.46 | 272.38 | 3.53 | 2 | 2 |

Criteria: TPSA≤140; MW ≤500g/mol; log P ≤5; H-bond acceptors N or O ≤ 10; H-bond donors NH or OH ≤5.

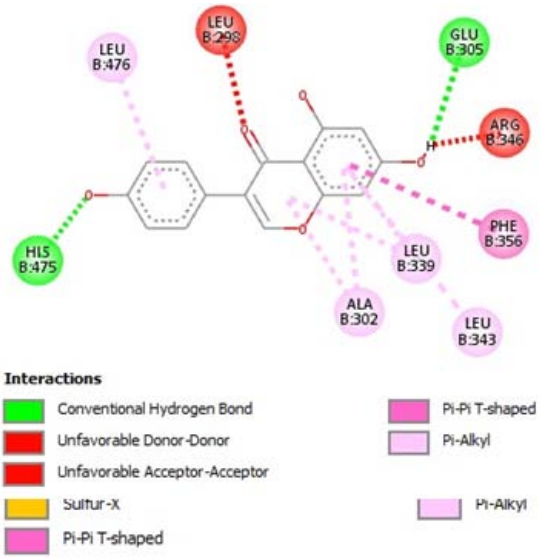


Fig. 4: (A) Binding mode of 17β-estradiol bond with the amino acid in the 3OLS protein. The 17β-estradiol binds amino acid with a pharmacophore distance (bond distance) of 10.862 Å, and binds his 475, Glu 305, and Arg 346 with hydrogen bonds. (B) Genistein binds amino acid with bond distance of 12.135 Å, and binds His 475 and Glu 305 with hydrogen bonds, and binds Arg 346 with an unfavorable bond

## DISCUSSION

The anti-neuroinflammatory effect of genistein occurs through the induction of inhibition of HMC3 microglia cells activation to a pro-inflammatory state ($M_1$ polarity), which is characterized by inhibition of MHC II expression. As well as induction of increase of HMC3 microglia cells activation to an anti-inflammatory state ($M_2$ polarity), which is characterized by an increase in Arg1 expression. Anti-neuroinflammatory activity was predicted to occur in an ER-dependent manner, so we also examined it using the ERβ as marker.

In this study, the model of inflammation-induced estrogen deficiency was made by induction of IFN-γ [17, 24, 25]. IFN-γ binds toll-like receptor 4 (TLR4) and activates nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB). Both estrogen deficiency and induction of IFN-γ on microglia HMC3 cells were known to be able to activate the cells to $M_1$ polarity conditions, where active microglia HMC3 cells have pro-inflammatory characteristics [7, 9].

Anti-neuroinflammatory activity occurs when genistein was given to microglia HMC3 cells as a treatment and replaces the function of estrogen in regulating the activity of the cell [11, 26-28]. In this process, there was an activation of microglia HMC3 cells to M2 polarity condition, where active microglia HMC3 cells have anti-inflammatory characteristics [7]. The increase of bonding between genistein and ERβ in the ER-dependent nuclear-initiated estrogen signaling pathway can activate ERβ in the cytoplasm to translocate in the nucleus, which causes several mechanisms such as inhibition of inhibitor of kappa B kinase (IκK) activation, inhibition of phosphorylation inhibitor of kappa B (IκB), inhibition of NF-κB activation, and induces down-regulation of the human leukocyte antigen B27 (HLA-B27) gene, which causes a decrease in the production of pro-inflammatory cytokines while production of anti-inflammatory agents is increase [2, 3, 5, 10].

The prediction of genistein activity can be carried out with *in silico* analysis. This method assists in simple predictions by correlating the structure and physicochemical properties with application analysis regarding its potential as medicine [29]. *In silico* studies were used to predict the activity of genistein in binding to ERβ at the molecular level, whether genistein is an ERβ agonist and is able to provide estrogenic effects such as 17β-estradiol or not. This *in silico* results were then used to explain the mechanism that occurs in the *in vitro* results.

The physicochemical properties of genistein were analyzed *in silico* based on the criteria of topological polar surface area (TPSA) value and Lipinski's rule of five. TPSA is a value that describes the ability of a compound to penetrate the membrane. The compounds are categorized as being able to penetrate the cell membrane if it has a TPSA value<140Å$^2$ [30]. From the physicochemical analysis, it was shown that genistein has TPSA value<140 Å$^2$, so it meets the criterion. The results of physicochemical analysis also indicate that genistein meets the criteria of Lipinski's rule of five, so it has the potential to be developed as an oral drug [31, 32].

In connecting the results of *in vitro* and *in silico* studies, we found something interesting. In the results of measuring the expression of Arg1 and MHC II *in vitro*, genistein has been shown to have anti-neuroinflammatory activity. Based on literature study and *in silico* results, this anti-neuroinflammatory activity occurs in an ER-dependent manner in which genistein bind to the ER. However, *in vitro* ERβ measurements showed that genistein could not increase the expression of free ER compared to control, that means genistein did not bind significantly to ERβ. Through a literature study, we found that genistein can bind to G-protein coupled estrogen receptor (GPER) with an effective and high-affinity bonding, better than to ERβ [33, 34]. Thus, the activity of decreasing MHC II expression and increasing Arg1 by genistein is likely caused by the binding of genistein to GPER. In microglia cells and neurons, GPER is highly expressed and is responsible for ER-dependent mechanisms of non-classical (or non-genomic) pathways [35, 36], such as anti-neuroinflammatory activity.

## CONCLUSION

Genistein shows anti-neuroinflammatory effects by decreasing the MHC II expression and increasing Arg1 expression in microglia HMC3 cell line, but the effect seems not to occur through the binding of genistein to ERβ. However, further study about the molecular interaction of genistein with other estrogen receptors is needed.

## FUNDING

Nil

## AUTHORS CONTRIBUTIONS

All the authors contributed equally.

## CONFLICT OF INTERESTS

Declared none

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