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RESEARCH ARTICLE

Effect of Ethyl Acetate Fraction of *Marsilea crenata* Presl. Leaf Extract on Major Histocompatibility Complex Class II Expression in Microglial HMC3 Cell Lines

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ABSTRACT:
Phytoestrogens are plant-derived chemical substances that have estrogen-like structures or estrogenic functions. Deficiency of estrogen in human brain causes neuroinflammation characterized by increase of major histocompatibility complex class II (MHC II) expression as a marker of M1 phenotype in microglia. Recent research found phytoestrogen compounds in *Marsilea crenata* Presl. The aim of this study was to investigate the effect of ethyl acetate fraction of *Marsilea crenata* Presl. leaf extract in MHC II expression of microglial HMC3 cell lines, for resolution of inflammation and tissue repair. The fractions were given at concentrations of 62.5, 125, and 250 ppm to microglia, that had been previously induced by IFNγ 10 ng for 24 hours to stimulate the cells into M1 phenotype. Genistein as phytoestrogen was given at a concentration of 50 μM as positive control. Expression of MHC II was analyzed using immunocytochemistry method. Result showed reduction in MHC II expression of microglial cells, which indicated the activity of all extracts and, showed that 250 ppm of the fraction showed the strongest effect with MHC II value expression of 148.632 AU, and ED50 of 1,590 ppm. It was concluded from the study, that ethyl acetate fraction of *Marsilea crenata* Presl. leaves has antineuroinflammation effect.

KEYWORDS: MHC II, Neuroinflammation, Phytoestrogen, *Marsilea crenata* Presl., Microglia.

INTRODUCTION:
Neuroinflammation is an inflammatory response of the brain mediated by major histocompatibility complex class II (MHC II), which is expressed by antigen presenting cells (APCs) of microglia[1].

They are molecules that have main function in presenting processed antigens from exogenous sources, and are expressed during the activation of microglia classical pathway to M1 polarity state. Polarity state is an active form of microglia cell which has proinflammation characteristics[2].

Estrogen deficiency among menopausal women is one of the causes of inflammation[3,4]. Treatment for estrogen deficiency-induced neuroinflammation include administering estrogen as hormone replacement therapy (HRT)[5]. However, long-term administration of HRT may lead to potential side effect[6], and therefore other alternatives which are safe and have minimum side effects, such as phytoestrogens, are needed[7,8,9].
Phytoestrogens are plant-derived chemical substances

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that have estrogen-like structures or estrogenic functions, and can replace endogenous estrogen for the functioning of all body organs to retain homeostasis[10,11,12]. From previous studies, it was known that *Marsilea crenata* Presl., one of popular plants from East Java Province of Indonesia, contain phytoestrogens. Some research showed that the leaves contain a high concentration of estrogen-like substances[13], and therefore could exert activities similar to endogenous estrogen, such as induction of bone formation, either in vitro in MC3T3-E1 cells, or in vivo in dexamethasone-induced mice[13,14,15,16]. Other studies showed, that *Marsilea crenata* Presl. contains secondary metabolites, such as flavonoids, polyphenols, steroids, alkaloids, and saponins[17,18,19].

The aim of the study was to understand the effect of ethyl acetate fraction of *Marsilea crenata* leaf extract on the inhibition of inflammation occured during activated M1 microglial state. Activation of the microglia was conducted by induction of IFNγ in microglial HMC3 cell lines for 24 h^2. MHC II expression as a marker of the M2 alternative state resulted from the shift of M1 state of microglial HMC3 cell lines would be identified by immunocytochemistry method using confocal laser scanning microscope (CLSM).

MATERIAL AND METHODS:

Plant Material:

Marsilea crenata Presl. leaves were collected from Benowo District, East Java Province, Indonesia, and determination was conducted by Center for Plant Determination, UPT Materia Medica, Batu, Indonesia. The leaves were sun dried and powdered.

Chemical:

Ethanol 96%, n-hexane, and ethyl acetate purchased from Merck, fetal bovine serum (FBS), paraformaldehyde (PFA), phosphate buffered saline (PBS), penicillin-streptomycin, genistein as positive control, Tween 80, anti-rabbit MHC II were purchased from Abcam, anti-rabbit fluorescein isothiocyanate (FITC), dimethyl sulfoxide (DMSO), eagle's minimum essential medium (EMEM) were purchased from Sigma-Aldrich.

Apparatus:

Confocal laser scanning microscopy (CLSM) Olympus Fluoview Ver.4.2a. software.

Extraction and Fractionation:

1,5 kg of *Marsilea crenata* Presl. powdered leaves were extracted with 96% ethanol, and fractionated with a mixture of 96% ethanol and 700 ml of water. Further fractionation was conducted using mixtures of n-hexane and ethyl acetate in a ratio of 1:1. Ethyl acetate fraction was then separated and evaporated by rotary evaporator Heidolph Hei-VAP ML/G3. Ethyl acetate dissolved flavonoids contents of *Marsilea crenata* Presl. leaves.

Cell Culture:

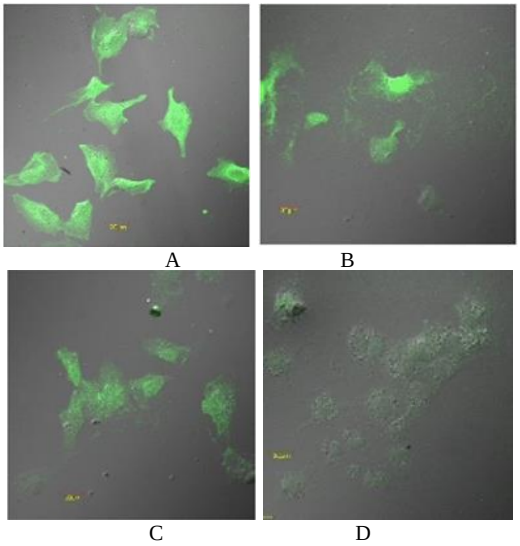
Microglial HMC3 cell line (ATCC CRL-3304) were purchased from American Type Culture Collection, USA. Cells were cultured in 24-well microplates in EMEM mixed with 10% FBS and 1% penicillin-streptomycin in a 5% CO2 incubator at 37°C for 6 days.

MHC II Measurement:

50 mg of ethyl acetate fractions were mixed with a 0.5% DMSO, and 0.5% of Tween 80, to prepare a suspension, and mixed with EMEM. The suspension was further diluted to obtain the desired concentration of 62.5, 125, and 250 ppm. Induction of IFNγ for 24 hours was conducted to cells that have been cultured on a 24-well microplate and reached 80% confluence. After induction, cells were rinsed with PBS, and mixed with the ethyl acetate suspension for another 48h incubation. After incubation, mixtures were fixated with 4% PFA. BSA and anti-rabbit MHC II were added and kept at 4°C for 24 h, and finally anti-rabbit FITC was added. The MHC II expression was analyzed using CLSM Olympus Fluoview Ver.4.2a. software at 488 nm. Data were analyzed by One Way Anova and Post-hoc LSD. Differences were considered significant at a significance level of $p<0.05$.

RESULT AND DISCUSSION:

This study was done using immunocytochemistry method to understand the antineuroinflammation activity through direct fluorescence antibody test[20]. Results were shown in Figure 1and 2.



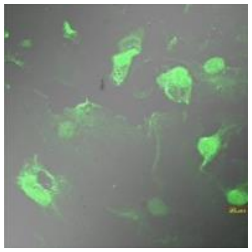


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Fig. 1. Immunofluorescence of HMC3 microglial cell, (A) Negative control group, (B) 62.5 ppm group, (C) 125 ppm group, (D) 250 ppm group (E) Positive control (Genistein) group.

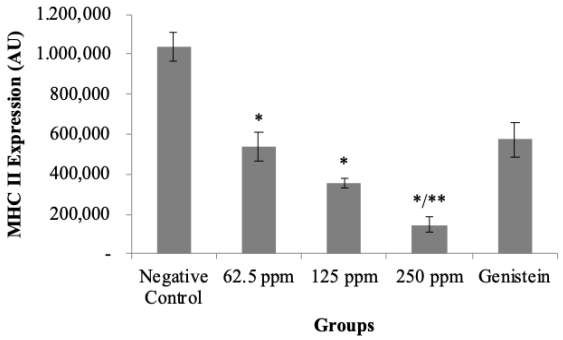

Fig. 2: MHC II Expression in microglial HMC3 cell lines after the administration of ethyl acetate fraction of *Marsilea crenata* Presl. leaves at concentrations of 62.5 ppm, 125 ppm, and 250 ppm. All fractions showed activities in lowering MHC II expressions which had significant differences compared to the negative control and positive control groups at $p < 0.05$. Strongest activity was found in 250 ppm fraction sample

Fig. 1 showed the immunofluorescence of HMC 3 cell lines in all groups. The strong intensity was found in negative control group, whereas the weak ones were shown in the genistein and treatment groups. This indicated, that genistein and treatment groups could reduce the expression of MHC II in microglial HMC3 cell lines. Microglial HMC3 cell lines of negative control group remained in M1 polarity, which was shown by its amoeboid-like morphology. Ethyl acetate fractions of treatment groups showed expressions that were significantly different compared to the negative control group. Treatment group of 250 ppm gave the lowest MHC II expression.

Fig. 2 described the quantitative results of MHC II expression of all groups. The ethyl acetate fractions of *Marsilea crenata* Presl. leaves of concentrations showed lower MHC II expressions compared to the negative control group. Interpretation of one-way ANOVA showed significant differences between the means of all treatment groups and the negative control at $p < 0.05$. ANOVA showed no significant difference between fraction of 62.5 ppm groups with positive control group. However, there is a significant difference between fractions of 125 and 250 ppm with genistein, in which both fractions were more active in reducing the MHC II expression than genistein. The strongest concentration was obtained by fraction of 250 ppm with the value of MHC II expression 148.632 AU. ED50 value of ethyl acetate fraction of *Marsilea crenata* Presl. leaf was 1.590 ppm. The value was calculated by statistical probit analysis of MHC II expressions of negative control and treatment groups.

Phytoestrogens are compounds produced in plants that can modulate the action of endogenous estrogens by binding to estrogen receptors. The compounds are known to have the ability to bind the estrogen receptors which is required for the ligand-receptor association[12]. Originally, the richest expression of ERβ is in the central nervous system, cardiovascular system, lungs, kidneys, urogenital tract, mammary glands, colon, and immune system. Genistein is also a phytoestrogen that showed a strong affinity for ERβ[21-28].

Lower blood estrogen level among menopausal women can cause various health problems, including inflammatory diseases, such as osteoporosis and neurodegenerative. The main cause of inflammation to occur is increasing production of cytokines, including TNF-α. As estrogen is known to have anti-inflammatory properties, hormone replacement therapy seems able to help in preventing inflammatory diseases associated with menopause[4,8,9].

Estrogen works by inhibiting NF-κB binding to the IL-6 promoter. Since NF-κB transcriptional factors activated inflammatory genes, it is supposed that estrogen blocks the inflammatory genes activation mediated by NF-κB. But, research found out, that actually estrogens exert anti-inflammatory effects through various mechanism. This can be explained through the fact, that estrogen did not repress the *TNF*-α gene by inhibiting the binding of NF-κB, but by recruiting steroid receptor coactivator 2 (SRC-2), which acts as a transcriptional repressor. This helps to understand that estrogenic drugs can have many targets responsible in the regulation of inflammatory genes. From such viewpoint, effort to find natural source of phytoestrogen will be helpful as alternative in replacing synthetic estrogens for hormone replacement therapy[27,29].

Previous research indicates the phytoestrogen contents of *Marsilea crenata* leaves. Phytoestrogens are bioactive compounds as secondary metabolites of plants that have therapeutic benefits[19,20,30]. They can modulate the action of endogenous estrogens by binding to estrogen receptors. The compounds are known to have the ability to bind to estrogen receptors which is required for ligand-receptor association. It is known that phytoestrogens could reduce the expression of MHC II

of microglia through estrogen receptor (ER) dependent pathway[10,11,12,31]. The binding between estrogen and ER can affects transcriptional gene through activated estrogen receptor (ER*). Transcriptional genes that are affected primarily was nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), which is a transcriptional factor responsible for immune system and inflammatory response[30]. Activation of NF-κB in microglial HMC3 cell lines will increase MHC II expression, and such activation occurred due to estrogen deficiency. Upon activation, NF-κB approached the nucleus and change the isomers of protein from monomers to oligomers which affect cell protein synthesis[32], such as MHC II. Therefore, an increase of ER* after administration of phytoestrogens could inhibit the activation of NF-κB, resulting in inhibition of MHC II expression and activation of microglia classical pathway to M1 polarity[10].

Microglia works through initiating inflammatory response to protect the body from any harmful stimuli, by producing various inflammatory cytokines such as TNFα, IL-6, IL-1β, and interferon-γ (IFNγ). Cytokines play a very pivotal role in the polarization process that will turn microglia into M1 activated state. In case of wound in the brain, inflammatory response has to be shifted into anti-inflammatory state for wound repairmen, and therefor proper transition from M1 to M2 phenotype is needed[2,33]

MHC II is a lipoprotein molecule that is important to regulate the immune response from extracellular pathogens. Increase expression of MHC II is caused by inflammatory inducing factors, such as IFNγ. As a result of IFNγ signaling, CD4+ T lymphocyte cells will be activated as a cellular specific immune system. CD4+ T lymphocyte cells will activate T helper 1 (Th1) cells, which in turn mediate MHC II expression as a proinflammatory marker in microglial cells. Th1 cells are responsible in immune response to intracellular pathogens, mainly through IFNγ secretion[33,34]. In this study inflammation occurred due to the induction of pro-inflammatory cytokine IFNγ in order to mimic the inflammation caused by estrogen deficiency. It aimed at activating microglial cells into M1 state[3,35].

It becomes clear from this study, that environment is critical in controlling the shift of M1 to M2 state, since prolonged inflammation could cause further neurodegeneration. Therefore, understanding M1/M2 dynamics is necessary, especially in diseases with chronic neuroinflammation[2,35].

CONCLUSION:

Ethyl acetate fraction of *Marsilea crenata* Presl. leaf extracts reduced MHC II expression in microglial HMC3 cell lines, due its phytoestrogen contents. This indicated the antineuroinflammation property of the phytoestrogens of the fraction. Further research is needed to investigate the specific types of phytoestrogen compounds.

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CONFLICT OF INTEREST:

The author states that there is no conflict of interest regarding the publication of this article.

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