ISSN 2338-0128
VOLUME 3

PROCEEDINGS BOOK
THE 7TH ANNUAL BASIC SCIENCE INTERNATIONAL CONFERENCE

7-8 March 2017
Ijen Suites Resort and Convention
Malang, Indonesia

Basic Science for Improving Survival & Quality of Life

Sub Topics:
Energy
Molecular and Health Science
Science and Technology Education

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Ethyl Acetate Fraction of Kalanchoe pinnata (Lmk)Pers Reduces the Production of IFN-α by Dendritic Cells in Pristane-induced Lupus-like Disease Mice

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Abstract – IFN-α has a central role in lupus pathogenesis. On the previous study, the ethyl acetate fraction of *Kalanchoe pinnata* (Lmk) Pers leaves (EF-KP) resulted in the modulation of the T regulatory function and the histology of the kidney. In this study, we observed the effect of the EF-KP on the IFN-α secreted by dendritic cells. The model used was Pristane-induced lupus-like disease mice according to Reeves method (2009) [1]. The experimental groups consisted of 3 groups, a positive control group that received placebo, EF-KP group, and negative control group that received cyclophosphamide. The treatment lasted for 3 weeks. At the end of the experiment, the semiquantitative method was used to observe the proteinuria as a general parameter. Then, the dendritic cells were isolated from the femur. The cells were measured by means of flow cytometry method. The proteinuria level of the positive control group was 79,00±33,81 mg/dL. The level of proteinuria of EF-KP group was 42,22±33,36 mg/dL, meanwhile, the negative control group had the level of 36,50±35,59mg/dL. This decrease indicates the repairing effect on of the kidney function. In the IFN-α measurement, we found the relative percentage of CD123+IFN-α+ reduced significantly in the EF-KP and the negative control groups. The result shows the reduction of the relative percentage of CD123+IFN-α+ in the EF-KP group. The decrease is lower than the positive control. It means that the EF-KP is able to inhibit the overexpression of the IFN-α which is produced by the dendritic cells. This inhibition could inhibit the lupus immune-pathogenesis [2], and also the proliferation of the autoreactive B and T cells.

Keywords: *Kalanchoe pinnata*, CD123+IFN-α+, lupus, proteinuria, treatment

1. INTRODUCTION

IFN-α has a central role in lupus pathogenesis. On the previous study, the ethyl acetate fraction of *Kalanchoe pinnata* (Lmk) Pers leaves (EF-KP) resulted in the modulation of T regulatory function and the histology of the kidney. According to the immunosuppressant activity in delayed-type hypersensitivity model which is reported by Bergmann (1994) [3] and in Pristane-induced lupus mice which are reported by Indriyanti (2011) [4], the EF-KP is suggested to influence on the IFN-α secretion. It also inhibits the inflammatory pathway indicated by using some biomarkers, such as TNF-α and TGF-β [5,6]. The early mechanism of Pristane is the effect on the plasmacytoid dendritic cells, which is dominantly present in the marrow-bone of the femur of the mice [2]. The dendritic cell markers are CD11c [1] and CD123. Therefore, this research purpose was to determine the effect of the EF-KP on the IFN-α which was secreted by dendritic cells in lupus mice.

2. METHODS

2.1 Materials

The *Kalanchoe pinnata* (Lmk) Pers fresh leaves were obtained from cultivation farm in Trenggalek, East Java. The botany identification of this plant was performed by Conservation Unit of Indonesian Institute of Science, Purwodadi with the identification number of 0284/IPH.06/HM/II/2015. The chemicals used in the fractionation were obtained from Merck through PT. Diamun as Indonesian supplier.

The female Balb/c mice aged 4 weeks were received from LPPT Gadjah Mada University, Indonesia. These mice were pathogen free species with the certificate number of 352/LP3HP/29/VII/2015. They were housed, randomized, and handled by using standard maintenance on the Guide of the Care and Use of Laboratory Animal 8th edition, published by National Research Council [7].

TMPD (Pristane) with the code number of Sigma-P2870 was obtained from the Sigma-Aldrich supplier at Singapore. Anti-CD123 and anti-IFN-α (Biogenesis), and also materials used in the flow cytometry assay were obtained from Biology Molecular Laboratory, Brawijaya University.

2.2 Procedures

The identity of the ethyl acetate fraction of *Kalanchoe pinnata* (Lmk) Pers (EF-KP) used in this research was provided as a chromatogram profile which was obtained by means of UPLC-QTOF-MS/MS tandem system. The UPLC instrument used was a UPLC Acquity SDS (Waters). The column used was Acquioty UPLC BEH C18 1.7um; 2.1x50 mm; flow rate 0.3 mL/minute. The injected volume was 5 uL (5000 ppm), and the temperature of 40°C. Eluants A: H2O+formic acid 0.1%; B: acetonitrile +formic acid 0.1%. The MS instrument used was XEVO-G2QTOF (Waters) with a positive ESI (resolution mode). The capillary voltage of 3 kV; sample cone voltage 38 V; desolvation T 300°C; source T 110°C; desolvation gas 500L/h; cone gas 16L/h. Then, the single compound profile was determined by means of densitometry method (Camag 3).

In this study, we observed the effect of the EF-KP on the IFN-α secreted by dendritic cells. The model used was Pristane-induced lupus-like disease mice according to Reeves (2009)[1]. The experimental groups consisted of 3 groups; a positive control group that received placebo, an EF-KP group, and a positive control group that received cyclophosphamide [8]. The treatment lasted for 3 weeks. At the end of the experiment, the semiquantitative method was used to observe the proteinuria as a general parameter. Then, the dendritic cells were isolated from the femur of the mice. The cells were measured by means of flow cytometry method. The data was analyzed by facilitating of BD CellQuest program. Finally, the data was calculated statistically by means of SPSS 22 version. The ethical clearance of this research was approved by local ICUC of Veterinary Medicine Faculty of Universitas Airlangga on January 12, 2016, with the certificate number of 326-KE.

3. RESULTS AND DISCUSSION

a. The identity of the EF-KP

Kalanchoe pinnata (Lmk) Pers has a lot of active compounds [9] which are predicted to result in many beneficially effects for lupus. The activities are immunosuppressant and anti-inflammatory [3,10], anti-allergy [11], immunomodulatory [12], and antioxidant [13]. No toxic effects occur during acute and subchronic toxicity test [14] and it is safe for the maternity since it could inhibit the uterus contraction [15-17].

The profile of the EF-KP which was tested in this research is shown in Fig 1.

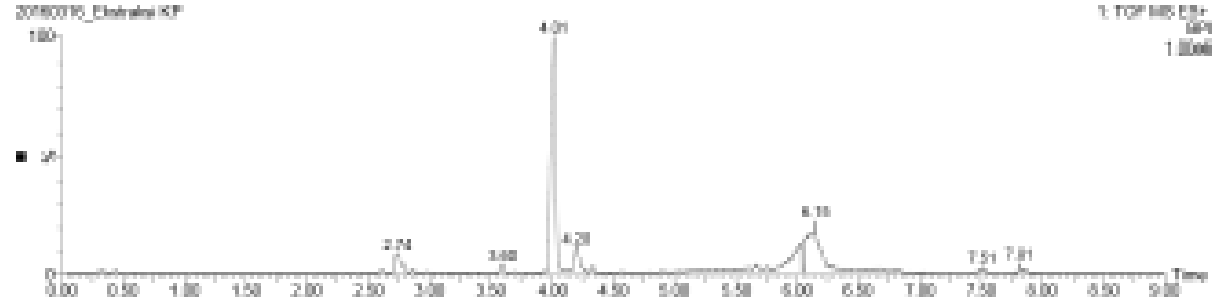

Fig 1. The chromatogram of the EF-KP

The liquid-liquid extraction (LLE) fractionation reduced a lot of compounds of the crude extract. According to the literature, the crude extract contains quercetin in the form of its glycosides. In the instrument, the glycosides are fragmented become its glycone and aglycone. The peaks at the retention time of 2.79 and 2.74 are predicted as the quercetin (aglycone). However, not all of the glycosides would be hydrolyzed well so that the active marker of rutin is better quantitated than the quercetin. The multiple peaks in the chromatogram made the quantitation of the marker invalid. In this research, we used the densitometry method to find the pure and single peak of rutin marker.

The rutin separation was performed based on the Indonesian Herbal Pharmacopeia procedure. The rutin spot was obtained on the HP-TLC plate (Silica gel 60 F_{254}) with the eluent of ethyl acetate P: n-butanol P: formic acid P (5:3:1). The EF-KP solution tested was 1.25% of the EF-KP in the ethanol P. The standard used was rutin 0.1% in the ethanol P, meanwhile, the detection reagent was sitroborat solution LP. The results of the densitometry assay are shown in Fig 2.

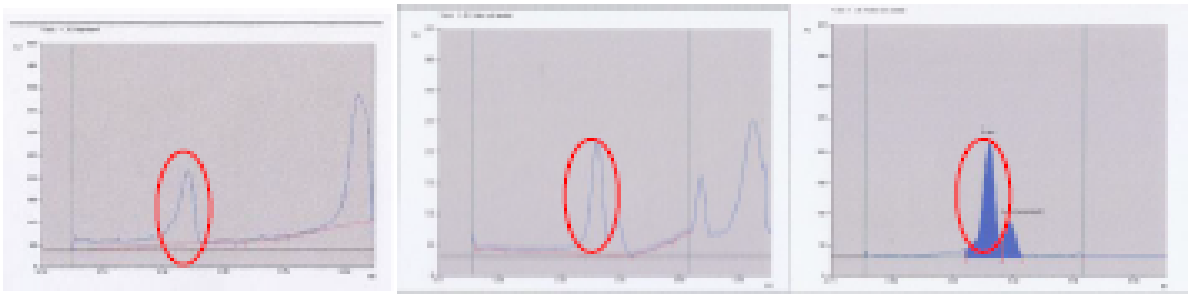

Fig 2. The rutin chromatogram of the rutin standard (a), EF-KP (b), and EF-KP (highlighted) (c)

The chromatograms were obtained by means of a densitometer (Camag 3). The results show the rutin peak in the EF-KP is not well separated to another peak. Thus, the optimization was continued so the quantitation of the rutin cannot be performed at present.

b. The *in vivo* experiment in lupus mice

This experiment was time-consuming because of the Pristane induction time that was lasted for 6 months to collect the mice which had the same baseline of proteinuria at the value of ++ (100 mg/dL) based on previous research [1,18,19]. The proteinuria level was the easiest parameter that could be observed without harming or killing the mice. Therefore, we use it as a general parameter which shows the kidney function repairing process in each experimental group. Then, the specific parameter observed was the CD123+IFN-α relative percentage. The data is described below.

The proteinuria level of the positive control group was 79.00±33.81 mg/dL. The level of proteinuria of EF-KP group was 42.22±33.36 mg/dL, meanwhile, the negative control group had the level of 36.50±35.59mg/dL. This decrease indicates the repairment of the kidney function.

Then, in the IFN-α measurement, we found the relative percentage of CD123+IFN-α+ reduces significantly (Fig 3) in the EF-KP and the negative control groups.

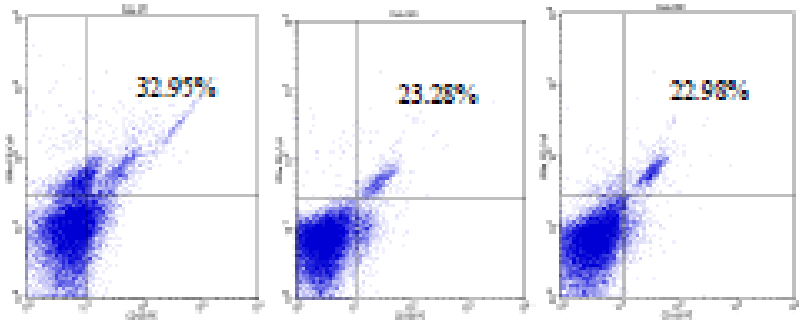

Fig 3. The profile of relative percentage of CD123+IFN-α+ analyzed by means of flow cytometry method in the femur of the positive control group (a), EF-KP group (b), and negative control group (c).

The result shows the reduction of the relative percentage of CD123+IFN-α+ in the EF-KP group. The decrease is lower than the negative control. It means that the EF-KP is able to inhibit the overexpression of the IFN-α produced by dendritic cells. This inhibition could inhibit the lupus immunopathogenesis [2], and also the proliferation of the autoreactive B and T cells.

Over-expression of the type I interferon, a kind of cytokine, usually correlates with a viral infection or autoimmune disease. There is a missing link between the type I interferon with a nucleic acid or antibody. However, the nucleic acid and the immune complex bind the *toll-like receptor* (TLR) lead to an innate immune activation. The effect is followed by the overexpression of IFN-I and IFN-II [20]. The IFN-α over-expression is mostly responsible for autoimmune pathogenesis [21,22].

Generally, the interferon which is correlated to the immune-modulation is IFN-gamma, meanwhile, the IFN-α is expressed dominantly in the viral infection. However, the Pristane effect demonstrates a different result. The IFN-α is the dominant interferon which impacts on many lupus biomarkers which result in some types of lupus-specific antibodies [1,2]. The IFN-α is secreted by some types of cells. According to the Pristane mechanism, the specific type of cell which products IFN-α is a dendritic cell. The dendritic cell surface markers are CD11c [1,22] and CD123. The cell is placed inside the femur bone.

This research is focused on the IFN-α inhibition by using the EF-KP as a drug candidate. The EF-KP activities such as anti-inflammation and antioxidant are the main consideration of the decision. The result shows the

reduction of the relative percentage of CD123+IFN-α+ with the value of 32.95±8.25% (placebo group), 23.28±9.31% (EF-KP group), and 22.98±10.39% (cyclophosphamide group). There is a significant (P<0.05) reduction of the relative percentage of the CD123+IFN-α+ in the group which received the EF-KP and another one which received cyclophosphamide. The result shows the EF-KP abilities to inhibit both the IFN-α and other biomarkers near the IFN-α pathway. Additionally, the production of antibodies would be decreased [23].

4. CONCLUSIONS

The EF-KP ability to inhibit the IFN-α pathway is predicted to be potential to inhibit the autoantibody production, causing the reduction of immune complexes. The further research is needed to observe the reverse effect caused by the immune-homeostasis mechanism.

5. ACKNOWLEDGEMENTS

This research was funded by DIPA PNBP 2015 Faculty of Pharmacy Universitas Mulawarman (Grant number 1787/UN17.12/HK/2015); Hibah Disertasi for Doctorate students 2016 (Grant number: 025/E3.4/2017) and BPPDN Scholarship for Indonesian doctorate student (Grant number: 906.8/E4.4/2015).

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