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THE ABILITY OF ANTI-INFLAMMATORY JATROPHA CURCAS LEAF EXTRACT AT COX-2 EXPRESSION ON MONOCYTES WERE EXPOSED LPS

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INTRODUCTION

Leukocyte is the main responsible cell in the immunity sistem which move freely in the circulation. Macrophag, neutrophil, dendrytic cell, mast cell, eosinophil, basophil and natural killer (NK cell) are important part of adaptive immunity. Macrophag is monocyte that leaves the circulation and move into the tissue to identify and directly kill the cause of the infection. Its killing mechanism is through the phagocytosis which responded to the pathogen bacteria, so the monocyte viabilty is important factor of the immune response.[1]

The interest of alternative medicine use in health therapy has been increased in the past few years.[2] Many researches has been conducted to discover the phytochemical of many herbs around the world, one of them is *Jatropha curcas*. Its leaves and branch contain *flavonoid, apigenin, vitexin, dan isovitexin*. It also contain dimer of triterpene alcohol ($C_{63}H_{117}O_9$) and two glycoside flavonoid.[3] In Indonesia, the Jatropha leaves has been used to cure the cough, as antiseptic, antipyretic, bloating relief, antihelminthics, gingivitis, stimulate hair-growth, anti-dandruff, antimalarial, and destruct the kidney stone.[4] Flavonoid has been known for its anti-microba and anti-viral activity. Triterpenoid also has the same activity and has been frequently found in herbs. It also helps the skin repair.[5] The extract of Jatropha leaves contain the flavonoid as the antiinflammatory agent.

In the previous study[6], the treatment with Jatropha leaves extract decreased the peripheral lymphocytes of Escherichia coli (E.coli)-induced male mice. Treatment with 5% concentration of Jatropha leaves extract shows the better effect compared to 10% when given on 1st until 7th day. Maghfirotin [7] stated the 10% concentration of Jatropha leaves extract also suppress the elevation of peripheral neutrophil in E.coli-induced male mice.

E.coli produce the well-know endotoxin, lypopolisacharide (LPS). Endotoxin has varies potention depend on the microorganisms, but its effect is quite the same.[8] LPS stimulates the immune cell through in vitro or in vivo environment, and has important clinical relevation because it is directly involved in Gram-negative bacteria mediated infection.[9]

Based on the introduction, the Jatropha leaves extract mechanism towards the COX-2 expression, the enzyme which responsible in inflammation phase, yet still unknown. This study is to determine the antiinflammatory activity of Jatropha leaves extract toward the COX-2 expression by using E. Coli to induce the inflammation.

MATERIALS AND METHOD

This in-vitro study use the posttest only with control group design. The independent variable is Jatropha leaves extract in 5%, 10% and 15 % concentration. The dependent variable is COX-2 expression on monocyte. The control variabel are monocytes type and concentration, LPS from E.coli and research procedures.

COX-2 expression was analyzed by immunohistochemical method. Monocyte isolation was incubated in Jatropha leaves extract and then it was exposed to LPS from E.coli. The cell was washed and then the immunostaining was performed use the antihuman COX-2 monoclonal antibody (Mab). The monocytes which expressed the COX-2 was observed and counted.

Making and Diluting the Jatropha leaves extract

1) The Jatropha leaves were collected from Faculty of Mathematics and Natural Science, Jember University. The chosen leaves was taken start from the fifth from the node. We took five leaves to uniform the sample.
2) The Jatropha leaves were identified in Herbarium Jemberience, Botany and Tissue Culture Laboratory, Nature Science Departement, Faculty of Mathematics and Natural Science Jember University.
3) The leaves were washed thoroughly, sliced in small portion and air-dried in room temperature for 24 hours without direct contact with the sunlight. The leaves were continued to dried into the oven for 3 hours in 45° C. When the drying process was finished, the leaves were ground in blender and sieved with 50 maze sieve. The results is 70 gram fine powder and was macerated with 95% ethanol for two days and stirred everyday. The solution was concentrated

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with rotary evaporator in 50ºC at 90 rpm speed. The 100% Jatropha leaves extract was finished and saved in the refrigerator. The extract was diluted into 5% and 10% concentration. The 5%, and 10% concentration was made in row by mixing 5 ml and 10 ml Jatropha leaves extract with 95 ml, and 90 ml distilled water.

Monocyte Isolate

This was done by ficoll hypaque centrifugation methods (Purwanto,2010).

1) 12 cc heparinized whole blood was divided into two, and was sentrifuged at 600 rpm for 10 minutes in room temperature.
2) The serum which contain the platelet was separated, and the remain was diluted in HBSS until reached 9 cc volume.
3) The falcon tube was prepared and filled with 3 cc ficol.
4) Carefully layer the diluted blood sample onto the Ficoll layer with disposible pipette.
5) Centrifuge for 30 minutes at 1400 rpm until the four layers were formed. From bottom to top (plasma, mononuclear, ficol and polynuclear red blood cell (+RBC)
6) Mononuclear layer contain mononuclear cells: lymphocyte and monocyte were separated and inserted into the falcon tube.
7) The cells were washed in HBSS and centrifuge at 600 rpm for 10 minutes, RT, 2 times to eliminate the platelet contaminant and resuspend the cells in 2500 µl HBSS with pippette.
8) Mononuclear suspension was layering on plastic microplate (24 wells; each well 100µl) which the base has already covered by cover slip. Incubate for 1 hour at 37oC.
9) The incubated medium contain lymphocite was discarded and the monocytes were washed thrice with HBSS.
10) Resuspended the monocyte pellet in RPMI 1cc for each well
11) Well was added by penstrep (5 µl dan fungison 5 µl), carefully medium pipeting, the monocytes were ready to incubated with Jatropha leaves extract.

Incubation with Jatropha Leaves Extract (JLE)

Monocyte isolate suspension was divided into four test groups (6 wells for each group):

1) K = control, not incubated with JLE, added by RPMI 1 cc.
2) P1= not incubated with JLE, added by RPMI 1 cc
3) P2= incubated with EDJP 5% (volume 1 cc)
4) P3= incubated with EDJP 10% (volume 1 cc)

Incubation was done in shaker incubator with 5% CO2, at 37°C for 18 hours.

LPS exposure

Monocyte isolate in group 2, 3, and 4 was exposed to 5µl LPS for each well and incubated for 1 hour at 370C with 5% CO2.

Immunostaning

1) Washed the monocyte suspension twice with HBSS
2) Fixate with absolute methanol for 1 minutes, and washed with aquades
3) Blocking, incubate for 5 minutes at room temperature.
4) Washed with PBS thrice, per 5 minutes.
5) Mab anti COX-2 (primary antibody) was applied with ratio 1:100 (10µ antibodi + 1cc PBSA), keep it for 24 hours at 4 0C.
6) Washed with PBS thrice per 5 minutes
7) Biotinylated Goat Anti-Polyvalent (secondary antibody) was applied, and incubated for 5 minutes at room temperature.
8) Washed with PBS thrice, per 5 minutes.
9) Streptavidin Peroxidase was applied, and incubated 5 minutes at room temperature
10) Washed with PBS thrice, per 5 minutes
11) Incubated with DAB chromogen stain (1,3-diamino benzidin), keep it for 10-20 minutes.
12) Washed with PBS thrice, per 5 minutes.
13) Washed with running water for 10-15 minutes.
14) Counterstain with Mayer's hematoxylin for 1-5 seconds.
15) Mounting

COX-2 Expression Parameter

COX-2 expression was showed on monocyte which has brown colour cell membrane, which observed from microscope with 1000x magnification. The result of this study is the mean of 100 cells which express the COX-2.

RESULTS

COX-2 expression was observed with microscope at 1000X magnification. The mean of 100 cells which express COX-2 and its standar deviation was shown in Table 5.1

Tabel 5.1 Mean ± SD of COX-2 expression on cell based on group

Group	Mean ± SD
Control	11,13 ± 3,18
LPS	18,27 ± 1,62
JLE 5%	13,07 ± 1,94
JLE 10%	8,87 ± 2,59

Keterangan: χ = Mean
Sd = Standar Deviation

The mean of COX-2 expression on K group 11,13 ± 3,18 ; LPS group : 18,27 ± 1,62 ; JLE 5% : 13,07 ± 1,94 ; and JLE 10% : 8,87 ± 2,59. This shows the COX-2 expression was higher in LPS group than the JLE 5% and 10% (Figure 5.1).

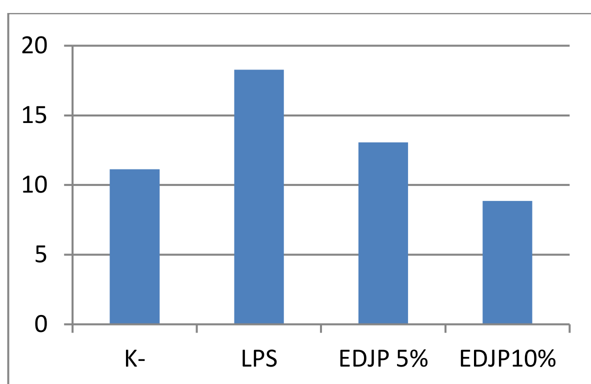

Figure 5.1 Bar graphic of COX-2 expression mean value on monocyte based on group

The highest COX-2 expression was found in LPS group, and the lowest was found in JLE 10%. The Kolmogorov- Smirnov was performed and the results was showed p>0,05 shows the data has normal distribution so the test was continued to Levene test, for homogenity test. The results was showed p>0,05 shows the data was homogen and continued by using One-way Anova. The result was showed in Table 5.2. p<0,05 shows there is significant difference of COX-2 expression between groups. To know which group has significant difference, the LSD test was performed. The results was showed in Table 5.3. p<0,05 shows there are significant difference between all groups.

Table 5.2. Result of One-way Anova between groups

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	723.000	3	241.000	41.484	.000
Within Groups	325.333	56	5.810		
Total	1048.333	59			

Table 5.3. Results of LSD test between groups

	Kontrol	LPS	EDJP 5%	JP 10%
Kontrol		**.000***	**.032***	**.013***
LPS			**.000***	**.000***
JLE 5%				**.000***
JLE 10%				

DISCUSSION

This study is in-vitro experiment using monocyte. Table 5.1 and 5.2 shows the COX-2 expression only found in group LPS, not in treated group with JLE and control. One-way Anova (Table 5.4) test showed the treatment of Jatropha leaves extract (JLE) has effect towards the decrease of COX-2 expression. It shows the 5% and 10% JLE inhibitory effect towards COX-2 expression in monocyte exposed to E.coli.

LPS is major constituent of gram negative-bacteria cell wall. LPS is endotoxin which induced the production of pro-inflammatory cytokine, such as interleukin 1α (IL-1α), IL-1β IL-6, tumor necrosis factor-α (TNF-α), and prostaglandin (PGE2).10 LPS activates the monocyte and macrophag to produce cytokines, adhesive molecule, pro-inflammatory mediator.9

COX expression was increasing in inflammation phase, both in COX-1 and COX-2. COX-1 is enzyme found in many cells, and COX-2 albeit in insignificant amount. COX-2 can be induced by cytokine, growth factor and other stimulus based on inflammation phase.11,12

COX-1 works in physiological condition, such as stimulating the mucous secretion to protect gastrointestinal tract mucous, hemostasis, wound healing, ovulation, and preserve kidney function.

COX-2 usually was expressed when the inflammation occured or in the other pathological condition.11,13 This study support this theory, that the highest expression of COX-2 was found in LPS group compared to other. JLE decrease the COX-2 expression in monocyte which has been exposed to LPS from E.coli because of its phytochemical though its mechanism is still not clearly unknown.

Jatropha leaves contain flavonoid, saponin, tannin and poliphenol. Flavonoid may inhibit the COX and lipoxygenase (LOX) which limit the migration of leukocyte and decrease the clinical sign of inflammation. Flavonoid also protect the cell membrane from the destructive agent.5 Inhibition of LOX may induce other effects such as as inhibition of eukasonoid product such as prostaglandin and tromboxan.5

Flavonoid is important to preserve the permeability and resistance of capillary. Flavonoid mostly works in endotelium microvascular to prevent the hyperpermeability and oedema. Flavonoid also inhibit the arachidonat acid release and the lisozyme release from neutrophils and endothels. Inhibition of arachidonat acid will decrease the substrate for COX and LOX pathway which the results is the supression of prostaglandin, prostacyclin, endoperoxid, tromboxan, hydroperoxid acid, dan leukotrien. This condition will affect the inflammation phase, such as leukocyte migration that will suppress the lymphocyte increase.14

Flavonoid almost has similar mechanism to aspirin by inhibiting the prostaglandin synthesis. It will inhibit COX irreversible, which responsible to catalyze the arachidonat acid to endoperoxide. With the proper dose, this will decrease the formation of prostaglandin and tromboxan A2.15 Saponin and tanin are natural product which can be found in herbs and has antibacteri, antiviral, antifungi activity to protect the host.16 When saponin interacts with

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bacteria, it will increase the membrane permeability of bacteria cell wall which induce the cell hemolysis.
In this study, the COX-2 expression in control group was found though there is exposure of LPS. We suppose it was the effect of bacteria contamination when the monocyte isolate was performed.
5% and 10% JLE decrease the COX-2 expression on the monocyte expose to LPS with twice higher decrease rate in the 10% JLE group. We supposed, the higher the JLE concentration, the higher the phytochemicals content. It may causes the inhibitory effect of COX and LOX will be stronger and also inhibit the COX-2 expression of monocyte which exposed to LPS.

CONCLUSION

Treatment with Jatropha leaves extract inhibit the COX-2 expression on monosit exposed to LPS, and the higher the concentration, the stronger the inhibitory effect on COX-2 expression on monocyte exposed to LPS.

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