Effect of *Pegagan* Leave (*Centella Asiatica* [L]. Urban) Ethanol Extract on IFN-γ Secretion on the Spleen of Balb/C Mice that Infected with *Listeria Monocytogenes*

IGMG. Surya C. Trapika[1]*, Mustofa[2], Eti Nurwening Sholikhah[2]

[1] *Pharmacology Dept., Faculty of Medicine, Udayana University*
[2] *Pharmacology & Therapy Dept., Faculty of Medicine, Gadjah Mada University*

** Corresponding author, email: suryatrapika@gmail.com*

Abstract

Pegagan *(*Centella asiatica *(L). Urban) is believed having immunostimulant potency; several related study have been done, though its effect on interferon gamma (IFN-γ) secretion have not been reported. Hence, this study aimed at exploring the effect of ethanol extract of* Pegagan *leave (*Centella asiatica *(L). Urban) on IFN-γ secretion on the spleen of Balb/C mice infected by* Listeria monocytogenes*. This quasy experimental study was conducted toward 50 female Balb/C mice that were grouped into five, namely: group K which was a control group that only given aquabidest; group EC-ZP given 1.95mg Echinacea and zinc pikolinat suspension; and group EEP I, EEP II and EEPIII that given ethanol extract of C. asiatica (L). Urban respectively at 50, 150 and 450mg/kgBW doses. The interventions were given for 14 days; on the day-15, all groups were infected with 104CFU* L. monocytogenes *intra peritoneal. Observation of IFN-γ secretion were performed at day-2 and 4 after infection. Results were analyzed by using analysis of variance to determine the statistical significance of IFN-γ secretion between groups. This study showed that ethanol extract of* C. asiatica *(L). Urban at 150mg/kgBW dose showed significantly higher IFN-γ secretion ($p<0.05$) compared to control group at day-4 after infection but has no difference with ethanol extract of* C. asiatica *(L). Urban at 50mg/kgBW dose. It can be concluded that at day-4 as peak of immune response of* L. monocytogenes *infection, ethanol extract of* C. asiatica *(L). Urban at 150mg/kgBW dose enhance IFN-γ secretion. Further study is needed to explore the effect of* C. asiatica *(L). Urban on other parameter of immune response related to IFN-γ secretion.*

Key words: Centella asiatica *(L). Urban,* Listeria monocytogenes*, immunostimulant, and IFN-γ secretion*

INTRODUCTION

Several plants that had been used for hundreds even thousands years by Egyptian, Greek, India and Chinese were believed to be beneficial to cure various diseases and also had been used as tonic for maintain healthiness. This ability is suggested to be related with the effect of those plants on the immune system, which then drives several researchers to study more extensively the active substance of the plants and its potency as immunomodulator(1)

Primary metabolite such as polysaccharide, manose, glukan, xylosa and fructose; and secondary metabolite such as flavonoid, antocyanin, alkaloid and terpenoid have effect on immune system.(2) Phytosterol produced by plants have certain role on improving lymphocyte T activity especially Th1 cell.(3). Moreover, saponin and triterpenoida in several plats have capacity to enhance activity of

immune mediator cell.[4,5] The effect of these metabolite to the immune response is through modulation the activity of dendritic cell, T and B lymphocyte cell, also the immune mediator such as interleukin, IFN dan tumor necroting factor (TNF).[6]

Pegagan (*Centella asiatica* [L]. Urban) can be found in humid area that is widely spread in tropical and subtropical area; it contains triterpenoid which are asiatikosida and madekasosida.[7] Several studies on *C. asiatica* (L).Urban showed it's potency as immunomodulator. *C. asiatica* (L). Urban is reported having effect on improving phagocyte index, IL-2 and TNF-α secretion, phagocyte mobility and activity of neutrofil and also *reactive oxygen intermediate* (ROI) secretion of macrophage.[8-10] Study by George *et al.* showed it's ability to hinder the degranulation of mast cell and its protective effect on mast cell which is even better than ketotifen fumarat.[11]

Several studies showed pharmacology activity of *C. asiatica* (L).Urban as immunomodulator has IFN-γ-like activity. Hence, the aim of the study was to learn the effect of *C. asiatica* (L). Urban extract towards IFN-γ secretion on the spleen.

Methodology

The study was a quasy experiment with posttest only control group design for exploring the effect of *C. asiatica* (L). Urban extract on IFN-γ secretion on the spleen. *C. asiatica* (L). Urban used in the study was obtained from PT. Indmira which is located at Pakem sub district, Sleman district, Yogyakarta, and it was determinated by Pharmaceutical Biology Department, Pharmaceutical Faculty, Gadjah Mada University, Yogyakarta. *C. asiatica* (L). Urban extract was produced through maceration method using 70% ethanol as the solvent. Animals used in the study were female Balb/C mice age 6-8 weeks and weight 25-30g, from Unit Pengembangan Hewan Percobaan LPPT, Gadjah Mada University, Yogyakarta.

In total, fifty mice were used in the study, divided into five groups with 10 mice in each group. Group K as control only received distillated water; Group EC-ZP received Imboost® (*Echinachea purpurea* dry extract 250mg and zinc pikolinat 5mg) at dose 0,0039 mL/0,5mL solvent; Group EEP I, EEP II and EEP III received ethanol extract of *C. asiatica* (L).Urban at different doses, namely at 50, 150 dan 450 mg/kg weight. The interventions were given for 14 days and at day-15 all groups were infected with bacteria *L. monocytogenes* at 10^4CFU, intraperitoneally. Bacteria were obtained from Balai Laboratorium Kesehatan, Yogyakarta. After infection, five mice from each group were sacrificed at day-2 and day-4.[12]

The mice were sacrificed with narcoses, the spleen was taken squeezed until single cell suspense in RPMI media was derived. Cell suspension was centrifuged at velocity 600 rpm and temperature 4°C for 10 minute. Then the pellet was re-suspensed into 2 mL tris buffer ammonium chloride to lyse the erythrocyte and were then centrifuged again at 600 rpm and 4°C for 10 minute. After centrifuge, the supernatant were discarded and lymphocyte was re-soluted with culture media (FBS, penicillin-streptomycin and fungizone). The number of cell was counted using hemocytometer and the viability was determined using tryptane blue ($1x10^6$/mL). A hundred micro liter cell suspension with the density $1x10^6$ was added into micro plate 96 wells with triplicate on each sample. On each samples were added 100µL culture media and phyto haemaglutinin (PHA) and three wells only contain culture media and PHA as controls. The cell was incubated in a CO_2 incubator at 37°C for 72 hours. After 72 hours, 100µL supernatant was collected for testing the IFN-γ secretion.

The measurement of IFN-γ level with ELISA *Sandwich* was using MaxDiscovery™ Mouse IFN-γ ELISA test kit, (Bio Scientific Corporation, USA); the

operational procedures are attached to the kit. Microplate was read using ELISA reader at wave length 450 nm. Then, the IFN-γ level was determined based on the value of OD into equation of standard curve.

Spleen lymphocyte cell was presented as mean ± SE and IFN-γ secretion was presented based on reflection of optical density value on the standard curve. For the comparison between groups, the analysis used was One Way ANOVA, that followed by Post Hoc analysis. Complete random group design test was applied for taking into account time of observation which then followed by *Duncan's new multiple-range test* (DMRT), the p value for significant result is if p value ≤0,05.5

RESULTS AND DISCUSSION

Effect of Ethanol Pegagan Extract on IFN-γ

At the first observation time, all three dose groups of ethanol extract of C. asiatica (L).Urban showed higher IFN-γ secretion than control group. The highest secretion was among group EEP II with mean secretion 17,420 ± 7,248 pg/mL. At the second observation time, most of the groups showed an increasing IFN-γ secretion except group EC-ZP, and the group with highest secretion was among group EEP I with mean 48,589±2,709 pg/mL.

Table 1. IFN-γ secretion (pg/mL) on each group after infected by *L. monocytogenes*

Observation (after infected by *L. monocytogene*)s	Experiment Group				
	K	EC-ZP	EEP I	EEP II	EEP III
Day-2	0,176±0,061	1,336±0,536*	14,497±7,721*	17,420±7,248*	3,254±1,616*
Day-4	2,129±0,793	1,206±0,806	48,589±2,709*	40,630±7,891*	3,505±0,609

*Note: K: control group which received distillated water; EC-ZP: received Imboost® (*Echinachea purpurea *dry extract 250mg and zinc pikolinat 5mg) at dose 0,0039mL; EEP I, EEP II, and EEP III: received ethanol extract of* C. asiatica *(L).Urban at 50, 150 dan 450mg/kgBW, respectively; *p < 0,05, compared to control group*

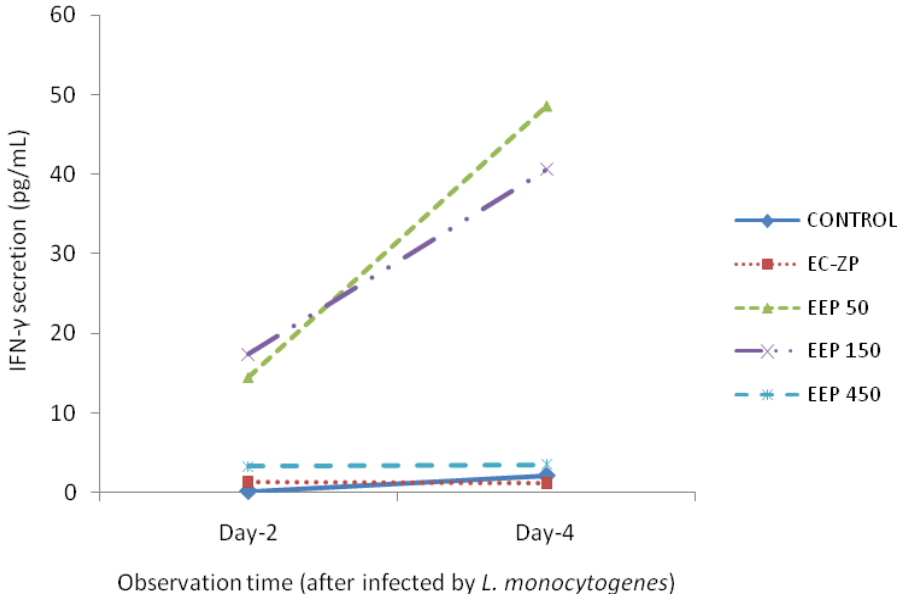

Figure 1. Configuration of IFN-γ secretion on each group.

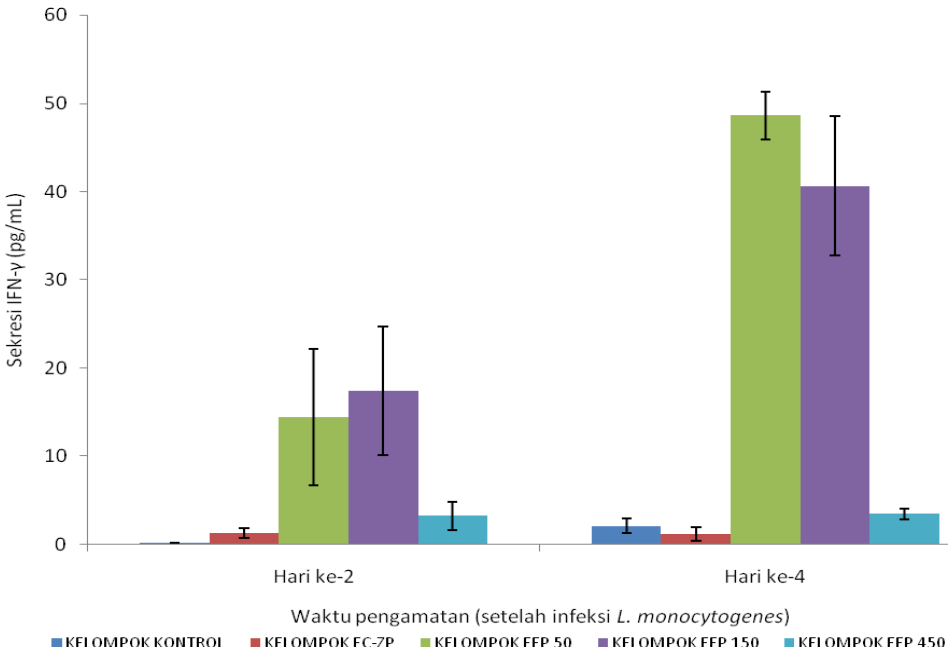

Figure 2. Graph of IFN-γ secretion on day 2 and day 4 after infection

Both One way Anova and complete random design test showed significant different on IFN-γ secretion between five groups on all observation times. Meanwhile, time and interaction between time and group did not show significant difference. Post hoc test with DMRT showed that ethanol extract of *C. asiatica* (L).Urban at dose 50 and 150 mg/Kg body weight did not differ significantly; while both doses were significantly different from the other three groups.

Table 2. Complete Random Design Test Result of lymphocyte IFN-γ secretion

Variable	F	Sig.
Group	16,429	0,000
Time	3,829	0,064
Group*Time	1,909	0,148

DISCUSSION

Invasion by pathogen will stimulate macrophage to produce TNF-α and IL-12. These cytokine stimulated natural killer cell (NK cell) to secrete IFN-γ. Synergy between IL-12 and IL-18, produced by macrophage will induce T lymphocyte to secret IFN-γ. That synergy also induce secretion of IFN-γ by antigen presenting cell (APC) and B lymphocyte.[13]

IFN-γ secretion mainly from Th1 lymphocyte, cytotoxic T cell, NK cell and some amount from macrophage, dendritic cell and B lymphocyte. On the intracellular bacterial infection such as by *L. monocytogenes,* T cell will mainly differentiated into T-CD8. There are two mechanisms on elimination of *L. monocytogenes* namely: secretion of perforin and granzime that will lyse the infected cell so the bacteria will be phagocited by active macrophage and secretion of IFN-γ will enhancing phagocyte activity of macrophage.[14] Secretion of IFN-γ can be detected on the spleen 16 hours after *L. monocytogenes* infection and reach the peak 48 hours after infection. It remain detectable until the fourth day after infection.[12]

The DMRT test signify that IFN-γ secretion on the control group only show no significant different with EC-ZP group, while with all ethanol extract of *C. asiatica* (L).Urban groups were significant. On this study, Imboost® doses were based on the recommended dose on packaging label which is 15 mL per day, it consist 250 mg dry extract of *E. purpurea* per 5 mL. On the other hand, The German Comission E recommended daily dose of *E. purpurea* at

900mg/day.[15] The capacity of ethanol extract of *C. asiatica* (L).Urban on stimulating IFN-γ secretion may explain its potencies that have been explored on previous studies. IFN-γ has certain role in the immune response such as activation of macrophage phagocytosis, differentiation of T cell into Th1 cell and blocking Th2 cell, regulation of chemokine that is related to attracting white blood cell include monocyte, eosinophile, basophile, and T cell, cellular adhesion molecules (VLA-4, ICAM-1, VCAM-1), suppress the Ig E formation, and enhancing Ig G synthesis.[19] Several pharmacologic effects of ethanol extract of *C. asiatica* (L).Urban include increase phagocyte activity of neuthrophil cell, ROI secretion of macrophage and anti allergy through blocking cell mast degranulation, may related to the ability of the extracts on stimulating IFN-γ secretion.

CONCLUSION AND RECOMMENDATION

From this study, it can be concluded that at day-4 after infection of *L. monocytogenes*, lymphocyte proliferation was higher on ethanol extract of *C. asiatica* (L). Urban doses 150 mg/kgBW than control group, and IFN-γ secretion was higher on ethanol extract of *C. asiatica* (L). Urban dose 150 mg/kgBW than control group.

Further studies is needed for exploring the effect of *C. asiatica* (L). Urban on other immune response parameters that related to lymphocyte proliferation and IFN-γ secretion mechanism.

ACKNOWLEDGEMENT

I would like to pass my gratitude to UPHP-LPPT Gadjah Mada University and Parasitology Laboratory Faculty of Medicine, Gadjah Mada University for the support during data collection.

REFERENCE

1. Licciardi PV, Underwood JR. Plant-derived medicines: A novel class of immunological adjuvants. Int. Immunopharmacol 2010;11:390-98.
2. Provenza FD, Villalba JJ. The role of natural plant products in modulating the immune system: An adaptable approach for combating disease in grazing animals. Small. Rum. Res. 2010;89:131-39.
3. Bouic PJD, Lamprecht JH. Plant sterols and sterolins: A review of their immunomodulating properties. Altern. Med. Rev. 1999;4(3):170-77.
4. Rajput ZI, Hu S, Xiao C, Arijo AG. Adjuvant effect of saponins on animal immune responses. J. Zhejiang Univ. Sci. B. 2006;8(3):153-161.
5. Mali RG, Hatapakki BC. An in vitro study of effect of *Centella asiatica* on phagocytosis by human neutrophils. Int. J. Pharm. Sci. Nanotechnol. 2008; 1(3):297-302.
6. Spelman K, Burns JJ, Nichols D, Winters N, Ottersberg S, Tenborg M. Modulation of cytokine expression by traditional medicines: A review of herbal immunomodulators. Altern. Med. Rev. 2006;11(2):128-150.
7. Zheng CJ, Qin LP. Chemical components of *Centella asiatica* and their bioactivities. J. Chinese Integr. Med. 2007;5(3):348-351.
8. Jayatirtha MG, Mishra SH. Preliminary immunomodulatory activities of methanol extracts of Eclipta alba and Centella asiatica. Phytomedicine 2004;11(4):361-65.
9. Punturee K, Wild CP, Kasinrerk W, Vinitketkumnuen U. Immunomodulatory activities of *Centella asiatica* and *Rhinacanthus nasutus* extracts. Asian Pac. J. Cancer Prev. 2005;6:396-400.
10. Akrom, Puspitosari D, Supriatno. Efek ekstrak etanol pegagan pada aktivitas sekresi reactive oxygen intermediates (ROI) makrofag peritoneal mencit Swiss betina diinduksi Spc-1. Jurnal Kedokteran Indonesia 2009;1(1):8-14.
11. George M, Joseph L, Ramaswamy. Anti-allergic, Anti-pruritic and anti-

inflammatory activities of Centella asiatica extracts. Afr. J. Trad. CAM 2009;6(4):554-59.
12. Nakane A, Numata A, Asano M, Kohanawa M, Chen Y, Minagawa T. Evidence that endogenous gamma interferon is produced early in Listeria monocytogenes infection. Infect. and Immunity 1990;58(7):2386-88.
13. Wong P, Pamer EG. Feedback regulation of pathogen-specific T cell priming. Immunity 2003;18:499-511.
14. Zenewicz LA, Shen H. Innate and adaptive immune responses to Listeria monocytogenes: a short overview. Microbes and Inf. 2007;9:1208-15.
15. Shah SA, Sander S, Whitw CM, Rinaldi M, Coleman CI. Evaluation of Echinacea for the prevention and treatment of the common cold: a meta analysis. Lancet Infect. Dis. 2007;7:473-80.
16. Teixeira LK, Fonseca BPF, Barboza BA, Viola JPB. The role of interferon-γ on immune and allergic responses. Mem. Inst. Oswaldo Cruz 2005;100(1):137-44.