In-vivo Immunomodulatory and Histophatological Effect of Two Compounds Isolated from Red Betel (*Piper crocatum* Ruiz & Pav.)

Yustina Sri Hartini[*1], Subagus Wahyuono[2], Sitarina Widyarini[3], and Ag. Yuswanto[2]
[1]Faculty of Pharmacy Sanata Dharma University, [2] Faculty of Pharmacy Gadjah Mada University, [3]Faculty of Veterinary Medicine Gadjah Mada University

*corresponding author :E-mail:yustinahartini@usd.ac.id; Tel:+62-8122968364,Fax:+62-274-886529

Abstract

Introduction

Red betel (*Piper crocatum* Ruiz & Pav) leaves have been used traditionally by Indonesian People to maintain their health. Previous study of the leaves indicated that the leaves displayed immunomodulatory activity *in vitro* (macrophage phagocytosis test). Two principal active substances were isolated from the MeoH Extract, identified as a neolignan (Pc-1) compound and its deacetyl derivative (Pc-2). This recent study we report histophatological effect and *in vivo* immunomodulatory effect on mice treated with Pc-1 and Pc-2.

Experimental

Assessment of immunomodulatory activity were carried out by macrophage phagocytic assay, nitric oxide assay, and lymphocytes proliferation assay. The material preparation was done by staining with hematoxylin-eosin, and the histophatological effects were investigated in liver and kidney of BALB/c mice induced by *Lysteria monocytogenes*.

Results & Discussion

At the dose of 5 and 10 mg/kgBW, both Pc-1 and Pc-2 increased activity and number of macrophages produced. Increasing of nitric oxide production due to Pc-1 and Pc-2 (2.5, 5, and, 10 mg/kgBW) was also observed even though there was not lymphocyte proliferation effect observed. Histopathological study of these two compounds did not show any abnormal histopathologic on liver and kidney upon Pc-1 treatment. But there was a liver damage was observed upon Pc-2 treatment although the kidney was still remained normal. Considering of this result and the structure similarity of these two compounds (Pc-1 and Pc-2), an interesting presumption can be brought up that the –OH functional group (Pc-2) was responsible for the toxicity causing liver damage.

Conclusion

The Pc-1 and Pc-2 isolated from the leaves of *P. crocatum* Ruiz & Pav. are able to increase the macrophages phagocytic and nitric oxide production activity as well, but lymphocytes proliferation effect is not observed. There is no histophatological effect on kidney observed due to both compounds, but histopathological effects on liver is observed due to Pc-2 treatment, not for Pc-1. Presumably, the –OH group on Pc-2 is responsible for the liver damage.

Key words : *Piper crocatum* Ruiz & Pav., histopathological, immunomodulatory, in vivo

INTRODUCTION

In recent years plant extract have been widely investigated for their possible immunomodulatory properties[1]. Research related to the application as an activator immunostimulatory immune system results are inconclusive and need a new immunostimulatory search of new sources[2]. Many plants used as traditional medicines reported to have immunostimulatory activity[3]. In Indonesia, red betel (*Piper crocatum* Ruiz & Pav.) was used as a medicinal plant for treating various diseases[4]. The red betel leaf extract inhibited proliferation of human breast cancer (T47D) cells[5]. In order to get the pharmacologically active compound two compounds wereisolated from the methanolic extract of red betel leaf. The compound are 2-allyl-4-(1'-hydroxy-1'-(3",4", 5 "-trimethoxyphenyl) propan-2'-yl) -3,5-dimethoxycyclohexa-3, 5-dienone (Pc-1) and 2-allyl-4-(1'-acetyl-1'-(3 ", 4", 5 "-trimethoxyphenyl) propan-2'-yl) -3,5-dimethoxycyclohexa- 3,5-dienone (Pc-2). *In vitro* phagocytic activity test of these two compounds showed immunostimulatory activity[6]. *In vitro* assay results do not always correlate with *in vivo* tests, it is necessary to confirm the *in vivo* test[2]. This study reports the results of Pc-1 and Pc-2 immunomodulatory effects on mice induced with *Listeria monocytogenes* ie: macrophage phagocytic, nitric oxide production, and lymphocyte proliferation test. Histopathological examination of liver and kidney was also performance.

EXPERIMENTAL

Plant material

The fresh leaves of red betel (*Piper crocatum* Ruiz & Pav.) were collected from Tawangmangu Central of Java, Indonesia on May 2010. Plant species was determined at department of Biology Pharmacy Faculty of Pharmacy, Gadjah Mada University, Yogyakarta, Indonesia.

Animals

Male Balb/c mice 8 weeks old weighing about 20-25 g and *Listeria monocytogenes* were used for the experiments. All procedures were approved by The Ethical Clearance Commision for pra-clinically research of Laboratorium Penelitian dan Pengujian Terpadu Gadjah Mada University, Yogyakarta, Indonesia. Balb/c mice were divided into nine groups. Group A, received 2.5 mg/kg BW Pc-1, Group B, received 5 mg/kg BW Pc-1, Group C, received 10 mg/kg BW Pc-1, Group D, received 2.5 mg/kg BW Pc-2, Group E, received 5 mg/kg BW Pc-2, Group F, received 10 mg/kg BW Pc-2, per oral for 14 days,

Group G, didn't received drugs as normal control, Group H, received 0.7 ml of 1% sodium carboxy methyl cellulose per oral as solvent control, and Group I, received 100 mg/kgBW. Product-X® (contain echinacea extract) per oral as positive control. On 15^{th} day (= day 0) and 25^{th} day 0.2 ml *L monocytogenes* containing $5x10^3$ cfu/ml are injected intraperioneally to all mice. On day 21 (37^{th} day) after infection the mice were sacrificed to take the macrophages and lymphocytes..

Compounds isolation

Red betel leaf methanolic extract was fractionated by vacuum liquid chromatography (VLC) method[7]. Isolated compounds (Pc-1 and Pc-2) is a purple spot at UV 254 nm, no color at UV 366, and brown on the cerium sulfate detection. It was eluated using chloroform : ethyl acetate (9:1) mobile phase with the retardation factor (Rf) of Pc-1 is 0:25 and the Rf of Pc-2 is 0.7.The Pc-1 and Pc-2 were isolated from 3^{th} and 4^{th} of VLC separation fractions using Thin Layer Chromatography (TLC) preparative. The band of the compound was scraped, collected, and then diluted with chloroform : methanol (1:1). The compound was obtained in the form of crystal after it was filtered and evaporated.

Phagocytosis assay

Latex beads was suspensed in PBS so that concentrations obtained 2.5 $x10^7$/ml. Medium taken by way of media suck so stay macrophages in coverslips. On the cover plate is identified by an appropriate test materials that will be included in the respective wells. At each well was added 1ml of test material in complete RPMI medium in triplo (3 coverslips) and then incubated for 4 hours. After 4 hours, suspension of latex beads added to PBS, where latex beads with a diameter of 0.3 lm at a density of 400 mL latex beads in 2.5 x107 / ml, then incubated in a CO2 incubator for 60 minutes. Media collected by aspirated and washed with PBS three times to remove the latex beads are not terfagositosis, then dried at room temperature and fixed with methanol for 30 seconds. Furthermore methanol removed and wait until dry and then painted with 20% Giemsa for 30 minutes, then washed with distilled water thoroughly (4-5 times, a clear distilled water) removed from the culture wells and dried at room temperature. Macrophages phagocytic latex particles are calculated using a light microscope[8,9,10] each at 2 field of view for each coverslip.

Nitric oxide (NO) assay

A total of 100 µL each macrophage cell cultures that have been incubated overnight, put in wells in duplicate, also the nitric oxide standard with 20µM concentration range up to 0.078 µM. Each wells was added 100 µL solution Gries (new made by mixing reagents Gries A: Gries B = 1:1). Incubated for 10 minutes, then read with elisa reader at a wavelength of 550 nm[11].

Lymphocytes proliferation test

Lymphocytes were cultured in microplate 96 with a volume of 100 mL/pitting. Each group with 3x replication. Added phytohaemaglutinin (PHA) at a final concentration of 50 ug/mL, 10 mL/wells, and then incubated at 37°C. The 3-(4,5-dimethyithiazol-2-yl)-2.5-diphenyltetrazolium bromide (MTT) is then added to each wells with a concentration of 5 mg / ml, 10 mL, and incubated at 37 ° C, 5% CO2 for 4 hours. The reaction was stopped by adding 0.04 M HCl-isopropanol as much as 100 mL/pitting. The result is read by ELISA reader at a wavelength of 550 nm[8].

Histopathological of liver and kidney

Murine peritoneum sheath is opened, after the isolation of macrophages and lymha, kidneys and liver removed and then immersed in a container of 10% formaldehyde solution until now making preparations histopathology. Fixation, dehydration, clearing, infiltration paraffin, and blocking (embedding) of the kidney and liver, the cutting is done with a thickness of 4 µm microtome, staining using hematoxylin-eosin (HE). Preparations examined under a microscope with a magnification of 100x[12].

Statistical Analysis

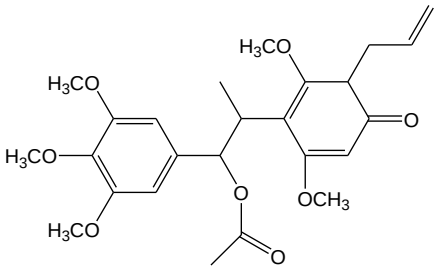
Phagocytic activity was measured by the latex-bead phagocytosis index (PI), the phagocytosis percentage (PP), and the phagocytosis efficiency (PE)[13]. Nitric oxide production and lymphocyte proliferation was measured by their absorbans using elisa reader. Expressed values are mean±SE using one way anova and Tukey test.

RESULT

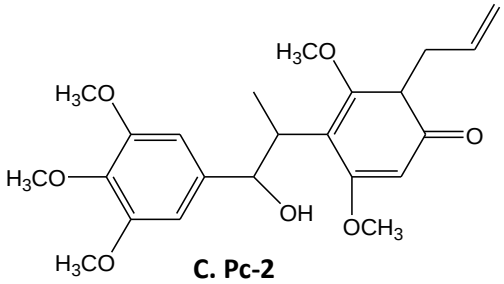
Isolation of Compounds

Extraction by maceration method using methanol to 1.9 kg of red betel leaf powder from drying produces 8.26 kg of wet leaf extract in the form of thick black mass as much as 224.03 g. The yield of powder on wet leaves by 23%, the yield on a wet leaf extract at 2.7%, and the yield of the extract powder of 11.8%[14]. Isolation of Pc-1 and Pc-2 from 2.12 grams of red betel leaf methanolic extract produced 12.0 mg of Pc-1 and Pc-2 12.1 mg. Compound Pc-1 and Pc-2 isolated from methanolic extract of red betel leaves are the same as Kustiawan's isolates. The Pc-2 differs from Pc-1 on their C1 binding group, Pc-1 bind the acetyl while Pc-2 bind the hidroksil.

A



B. Pc-1



C. Pc-2

Fig. 1: A. Red betel (*Piper crocatum* Ruiz & Pav.), B. & C. The two compounds from red betel (*Piper*

Immunomodulatory Effect

Both compounds were isolated from the leaves of red betel (*Piper crocatum* Ruiz & Pav.) increase the phagocytic activity and capacity of peritoneal macrophages of mice infected with *Listeria monocytogenes*.

Treatment with compound Pc-1 and Pc-2 at a dose of 5 or 10 mg/kgBW showed a significant difference in NO production compared to treatment with normal control, solvent control and positive control whereas at a dose of 2.5 mg/kgBW showed significant differences to the three control groups..

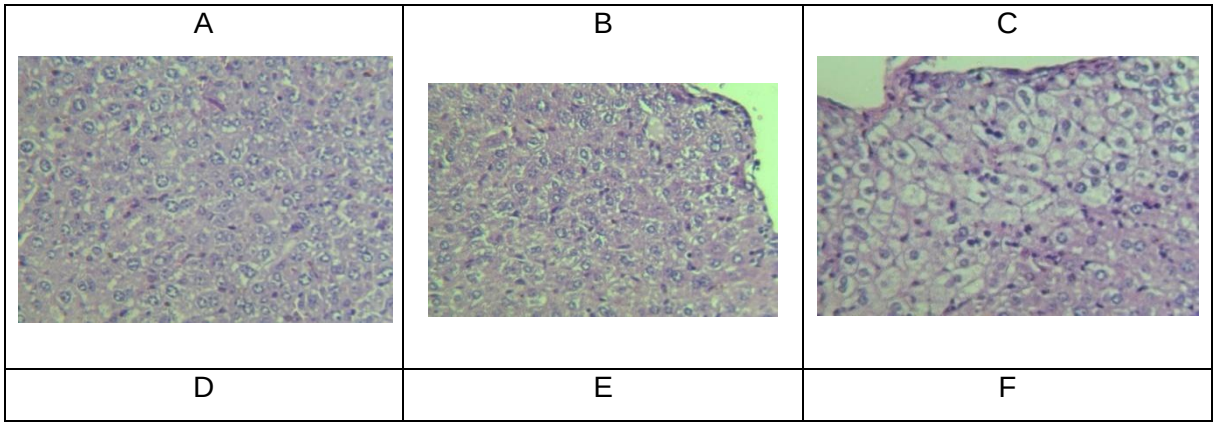
Lymphocyte proliferation assay showed no significant difference among Pc-1 and Pc-2, respectively at doses of 2.5, 5, and 10 mg/kgBW compared to solvent control and medium control group ($P<0.05$).

Table 1: Immunomodulatory effect of Pc-1 and Pc-2 on the 21th day after the mice were infected with *L. monocytogenes*

Group	Percent Phagocytosis	Index Phagocytosis	Efficiency Phagocytosis	NO Production	Lymphocyte Proliferation
Pc-1 (2.5 mg/kgBW)	17.2 ± 0.5	26.9 ± 1.1	1.57 ± 0.03	0.077 ± 0.000*	0.069 ± 0.001
Pc-1 (5 mg/kgBW)	25.1 ± 2.6*	38.2 ± 3.6*	1.53 ± 0.01	0.081 ± 0.000*	0.056 ± 0.006
Pc-1 (10 mg/kgBW)	37.5 ± 1.8*	61.1 ± 2.9*	1.63 ± 0.01	0.085 ± 0.001*	0.056 ± 0.007
Pc-2 (2.5 mg/kgBW)	18.2 ± 1.6	29.6 ± 3.9	1.65 ± 0.26	0.078 ± 0.000*	0.061 ± 0.001
Pc-2 (5 mg/kgBW)	22.8 ± 0.7*	52.2 ± 3.2*	2.29 ± 0.07*	0.079 ± 0.000*	0.065 ± 0.003
Pc-2 (10 mg/kgBW)	42.9 ± 2.2*	96.9 ± 7.4*	2.26 ± 0.11*	0.086 ± 0.000*	0.056 ± 0.004
Normal control	9.8 ± 0.3	13.1 ± 0.8	1.35 ± 0.08	0.070 ± 0.000	0.036 ± 0.012
Solvent control	9.7 ± 0.2	13.5 ± 0.7	1.40 ± 0.04	0.070 ± 0.001	0.039 ± 0.013
Positive control	21.4 ± 3.3*	39.6 ± 9.2*	1.81 ± 0.13	0.078 ± 0.001*	0.053 ± 0.003

Values are presented as mean±SE of three mice, *P<0.05 considered to be significant difference,when compared to the normal control and solvent control

Histopathological Effect on liver and kidney



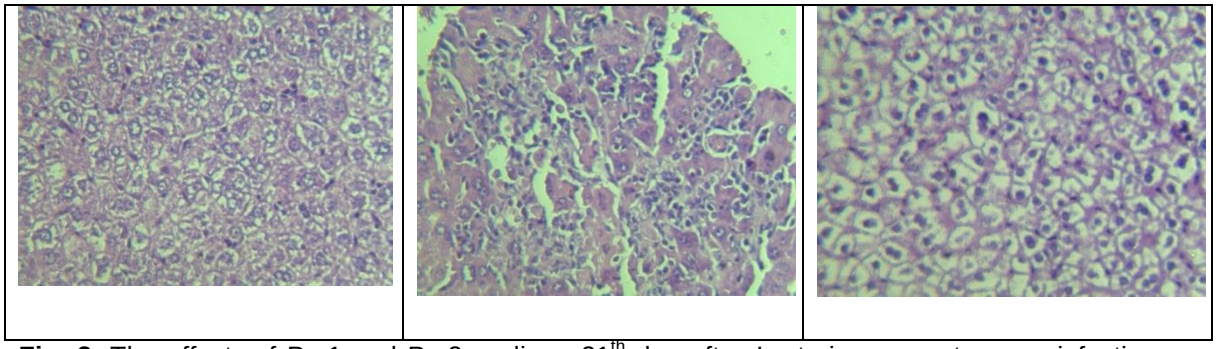


Fig. 2: The effect of Pc-1 and Pc-2 on liver, 21th day after *Lysteria monocytogenes* infection on BALB/c mice. A: medium control, B: solvent control, C: positive control, D: 2.5 mg/kg BW Pc-2, E: 5 mg/kg BW Pc-2, F: 10 mg/kg BW Pc-2. Pc-1 (2.5, 5, 10 mg/kg BW) = A = B

There was differences on histological profile because of 14 days *in vivo* treatment of Pc-1 and Pc-2. All group that received Pc-1 at doses of 2.5, 5, and 10 mg/kg BW showed normal histological kidney and liver, while group that received Pc-2 showed normal histological kidney but abnormal in liver. There was a mild hidropic degenerative liver because of *in vivo* treatment at dose 2.5 mg/kgBW, necrotic liver on centro lobules and mild locally hidropic degenerative liver because of *in vivo* treatment at dose 5 mg/kgBW, and a severe (diffused) hidropic degenerative with locally necrotic liver because of *in vivo* treatment at dose 10 mg/kgBW Pc-2.

DISCUSSION

Compounds Pc-1 and Pc-2 showed the same pattern of effects on macrophage phagocytic activity and capacity. At a dose of 10 mg/kgBW both Pc-1 and pc-2 is able to increase the activity of macrophages and capacity. Trials showed statistically significant difference ($P<0.05$), the percent phagocytosis and phagocytic index of macrophage groups of mice treated with 10 mg/kgBW Pc-1 BB against the normal control group and the solvent control, as well as to the positive control group-X product containing 250 mg of Echinacea extract. The ability echinacea extract in enhancing phagocytosis has been reported by Bauer (1999). At a dose of 5 mg / kg both Pc-1 and Pc-2 is able to increase the activity and capacity of macrophages significantly different both in the normal control group and the solvent control, but not significantly different from the positive control group. At a dose of 10 mg/kgBW both Pc-1 shows significant differences dose of 10 mg/kgBW both Pc-2 on phagocytic index parameter. At a dose of 10 mg / kg Pc-2 macrophage phagocytic capacity greater than Pc-1 at the same dose. to phagocytosis efficiency parameter, Pc-2 at

a dose of 5 mg/kgBW or 10 mg/kgBW showed a significant difference whereas Pc-1 only at a dose of 10 mg kgBW.

Altough thre is a statistically different, the NO level is low, with the number ± 0.08 µM, while the acticity and capacity of phagocytosis is relative high, it seems that the phagocytic mecanisme action do not via NO production, other mechanisms like myeloperoxidase

There was no effect of both Pc-1 and Pc-2 on the lymphocytes proliferation, Apriyanto (2011) and Indriyani (2011) also reported that extract of *Piper crocatum* Ruiz & Pav. did'n effect on lymphocyte proliferation. Table 1 showed that increases of doses tend to reduce the lymphocytes proliferation, it seem that *Piper crocatum* Ruiz & Pav have immunosupression activity, because Kanjwani *et al.* (2008) reported that methanolic extract of *Piper betle* L.; other species on the same genus of *Piper crocatum* Ruiz & Pav.; gave immunosupressan on cellular and humoral response, moreover Yuristiyani (2012) found that *in vitro* assay showed it suppres the lymphocytes proliferation.

Since Pc-1 and Pc-2 difference in an acetyl group of their structure, it could be the functional group that responsible for the abnormality of the liver. *Piper crocatum* Ruiz & Pav. is an interesting natural product wich two similar compounds that have the same immunomodulatory activity but different liver histological architecture. Compound Pc-2 wich caused liver abnormality is an acetyl of Pc-1, their effect on macrophage phagocytic, nitric oxide production, and lymphocyte proliferation is similar, the acetyl could be the functional group that responsible for the abnormality of the liver.

Tirapelli *et al.* (2011) suggested that increases in NO production may be an early indicator of liver damage, but in this research abnormality of liver do not correlate to increases of NO generation because both Pc-1 and Pc-2 increase in NO production but damaged of liver just happened because of Pc-2.

Since 2.12 g *Piper crocatum* Ruiz & Pav. methanolic extract contains 12.1 mg Pc-2 or doses 2.5mg/kgBW Pc-2 equal to 438mg/kgBW extract. Wiweko (2010) found that ethanolic extract *Piper crocatum* Ruiz & Pav. leaf at dose 300mg/kgBW didn't effect on histophatological kidney and liver, moreover Wahyudi (2010) found that the same dose of hexane extract *Piper crocatum* Ruiz & Pav. leaf didn't effect on histophatological kidney

CONCLUSION

The two compounds (Pc-1 and Pc-2) were isolated from *Piper crocatum* Ruiz & Pav. had increased the macrophages phagocytic and nitric oxide production activity but had no

effect on lymphocytes proliferation. Both compounds have no histophatological effect on kidney but Pc-1 showed a better histopathological effects on liver than Pc-2.

ACKNOWLEDGMENT

The author thanks to Direktorat Jenderal Pendidikan Tinggi Republik Indonesia for providing financial support for this research by hibah disertasi doktor.

REFERENCES

1. Alamgir M.; Uddin SJ. Recent advanced on the etnomedicinal plants as immunomodulatory agents. In: Debprasad Chattopadhyay,editor.Ethnomedicine: a source of complementary therapeutics. Kerala: Research Signpost; 2010. p 227-244.
2. Wagner H.; Kraus S.; Jurcic K.;. Search for potent immunostimulating agents from plants and other natural sources. In: Wagner H., editor. Immunomodulatory agents fromplants. Basel: Birkhauser Verlag; 1999. p.3-4.
3. Bafna A.R. dan Misrha S.H. Imunomodulatory activity of methanol extracts of flower-heads of *Sphaeranthus indicus* Linn. Ars Pharm 2004;45(3):281-291.
4. Manoi F.; Sirih merah sebagai tanaman obat multi fungsi. Warta *Pusat Penelitian dan Pengembangan Perkebunan* 2007;*13:2-6.*
5. Wicaksono BD.; Handoko YA.; Arung ET.; Kusuma IW.; Yulia D.; Pancaputra AN.; Sandra F. Antiproliferative effect of methanol extract of *Piper crocatum* Ruiz & Pav leaves on human breast (T47D) cells *in-vitro*. Tropical Journal of Pharmaceutical Research 2009;8:345-352.
6. Kustiawan PM. Isolasi dan identifikasi senyawa imunostimulan non spesifik *in vitro* dari daun sirih merah (*Piper crocatum* Ruiz & Pav.). [Thesis].Yogyakarta.Faculty of Pharmacy Gadjah Mada University. 2012. p 30-45.
7. Coll JC; Bowden BF. The apllication of vacuum liquid chromatography to the separation of terpene mixtures. Journal of Natural Products 1986;49:934-936.
8. Leijh PCJ.; Furth RV.; Van Zwet TL. In vitro determination of phagocytosis and intracellular killing by polymorphonuclear and mononuclear phagocytes.In: Weir DM, editor. Cellular Immunology.4th ed. Oxford:Blackwell Scientific Publications;1986. p 46.1-11.
9. Wahyuniari IAI. pengaruh pemberian minyak buah merah (*pandanus conoideus* lamp) pada respon imun seluler setelah infeksi *Listeria monocytogenes*. [Thesis].Yogyakarta.Faculty of Medicine Gadjah Mada University. 2006 June. 24-27 p
10. Derre I.; Isberg RR. Macrophages from mice with the restrictive lgn1 allele exhibit multifactorial resistance to *Legionella pneumophila*. Infect. Immun 2004;72(11): 6221-6229.
11. Cheng A.; Wan F.; Wang J.; Jin Z.; Xu X. Macrophage immunomodulatory activity of polysaccharides isolated from *Glycyrrhiza uralensis* fish. International Immunopharmacology 2008; 8:43-50.
12. Lynch MJ.; Raphael SS. Mellor LD.; Spare PD. Medical laboratory technology and clinical pathology. Philadelphia, Saunders Company WB; 1969; 987-1231p.
13. Sanchez S.; Paredes SD.; Sanchez CL.;Barriga C.; Reiter RJ.; Rodriquez AB.; Tryptophan administration in rats enhances phagocytic function and reduces oxidative metabolism. *Neuro Endocrinol Lett*. 2008;Dec:29(6):1026-1032.

14. Hartini YS.; Wahyuono S.; Widyarini S.; Yuswanto A. Uji aktivitas fagositosis makrofag ekstrak metanolik daun sirih merah (P*iper crocatum* ruiz & pav.) secara *in vitro,* seminar pemberdayaan pasien dalam self management diabetes melitus untuk meningkatkan kualitas hidup. [*Proceeding*]. Faculty of Pharmacy Sanata Dharma University Yogyakarta. 2011: p.20-28
15. Bauer R. Chemistry, Analysis and immunological investigations of echinachea phytopharmaceuticals, In: Wagner H, editor. Immunomodulatory agents from plants. Basel: Birkhauser Verlag;1999. p.41-89.
16. Apriyanto S. Uji aktivitas imunomodulator ekstrak etanolik daun sirih merah (*Piper crocatum* Lamk) terhadap proliferasi sel limfosit dan fagositosis makrofag pada tikus yang diinduksi vaksin hepatitis B, [Skripsi].Yogyakarta.Faculty of Pharmacy Gadjah Mada University. 2011 July. 55 p
17. Indriyani LW. Uji Aktivitas imunomodulator ekstrak n-heksana daun sirih merah (P c Lamk) terhadap aktivitas fagositisis makrofag dan proliferasi sel limfosit tikus yang diindusksi vaksin hepatitis B, [Skripsi].Yogyakarta.Faculty of Pharmacy Gadjah Mada University. 2011 May. 67 p
18. Kanjwani DG.; Marathe TP.; Chipunkar SV.; Fan Sathaye SS. Evaluation of immunomodulatory activity of methanolic extract of *Piper betel*. *Scand J of Imm.* 2008;67:589-593.
19. Yuristiyani DD. Potensi fraksi tak larut n-heksana ekstrak etanol daun sirih merah (*Piper crocatum* Ruiz & Pav.) secara *in vitro* sebagai imunomodulator terhadap fagositosis makrofag dan proliferasi sel limfosit tikus yang diinduksi phytohemagglutinin, [Skripsi].Yogyakarta.Faculty of Pharmacy Gadjah Mada University. 2012 Sept. 52 p
20. Tirapelli LF; Batalhao ME.; Jacob-Ferreira AL; Tirapelli DP.; Carnio EC.; Tanus-Santos JE.; Queiroz RH.; Uyemura SA.; Padovan CM.; and Tirapelli CR.; Chronic ethanol consumption induces histopathological changer and increases nitric oxide generation in the rat liver, Tissue and Cell 2011; 43: 384-391.
21. Wiweko OD. Uji aktivitas imunomodulator ekstrak etanol daun sirih merah (*Piper crocatum* Lmk) terhadap peningkatan titer imunoglobulin g dan histopapatologi tikus yang diinduksi vaksin hepatitis B. [Skripsi].Yogyakarta.Faculty of Pharmacy Gadjah Mada University. 2010 Dec. 46 p
22. Wahyudi, Y. Uji aktivitas imunomodulator ekstrakn-heksana daun sirih merah (*Piper crocatum* Lmk.) terhadap peningkatan titer imunogobulin g pada tikus terinduksi vaksin hepatitis b dan uji histopatologinya. [Skripsi].Yogyakarta.Faculty of Pharmacy Gadjah Mada University. 2010 Dec. 44 p