PROCEEDING

THE 3rd INTERNATIONAL CONFERENCE ON

PHARMACY AND ADVANCED PHARMACEUTICAL SCIENCES

Book 1:
Pharmaceutical Science & Technology

Faculty of Pharmacy
Universitas Gadjah Mada
Yogyakarta, June 18-19, 2013

Universiteit Utrecht

CYBERJAYA UNIVERSITY
COLLEGE of MEDICAL SCIENCES

Table of Content

May 2010. Plant species was determined at department of Biology Pharmacy Faculty of Pharmacy, Gadjah Mada University (GMU), Yogyakarta, Indonesia.

2. Animals and microorganisms:

Male Balb/c mice 8 weeks old weighing about 20-25 g obtained from Pharmacology Laboratory while *Listeria monocytogenes* obtained from Microbiology Laboratory, Faculty of Medicine GMU were used for the experiments. All procedures were approved by The Ethical Clearance Commision for pra-clinically research of Laboratorium Penelitian dan Pengujian Terpadu GMU. Balb/c mice were divided into nine groups. Group A, received 2.5 mg/kgBW Pc-1, Group B, received 5 mg/kgBW Pc-1, Group C, received 10 mg/kgBW Pc-1, Group D, received 2.5 mg/kgBW Pc-2, Group E, received 5 mg/kgBW Pc-2, Group F, received 10 mg/kgBW Pc-2, per oral for 14 days, Group G, didn't received drugs as normal control, Group H, received 0.75 ml of 1% sodium carboxy methyl cellulose per oral as solvent control, and Group I, received 100 mg/kgBW Produk-X® per oral as positive control. On 15th day (=day 0) 0.2 ml *L. monocytogenes* containing $5x10^3$ cfu/ml are injected *intraperioneally* (ip) to all mice. On day 10, three mice of each group were used to obtaine 10th day data, while 3 mice letf of all group are injected ip again with 0.2 ml *L. monocytogenes*. These mice are sacrificed on day 21 to obtain 21th day data.

3. Chemicals:

Medium *Rosewell Park Memorial Institute* (RPMI)-1640 (Sigma R-6504), *fetal bovine serum* (FBS) (Gibco BRL 10082147), 1mM sodium bicarbonate ($NaHCO_3$) (Sigma S-5761), mM L-Glutamin 100, µg penisilin, dan 0.5 mg streptomisin (Gibco 15140-122), fungison (Gibco 15295-017), *Phosphate-buffered saline (PBS),* chloroform (Merck K 23788209709), methanol (Merck K 2538245), *latex beads* (Sigma LB-30) diameter 0,3µm, Giemsa 10%, *tryphan blue,* Product-X® (kaplet Imboost Force PT Kimia Farma).

4. Methods:

4.1. Compounds isolation.

Red betel leaf methanolic extract was fractionated by the method of vacuum liquid chromatography (VLC) (Coll and Bowden, 1986) successively with n-hexane, chloroform, ethyl acetate, and methanol so that the final fraction obtained 5. Isolated compounds (Pc-1 and Pc-2) are compounds with a purple marker spots that reduce UV_{254}, no color at UV_{366} nm, and brown on the detection of cerium sulfate, with a mobile phase of chloroform: ethyl acetate (9:1). The Pc-1 (Rf 0:25) and Pc-2 (Rf 0.7) contained in fraction III and fraction IV results VLC separation of methanolic extract of red betel leaf. Fraction was dissolved in chloroform: methanol (1:1) and then spotted on preparative TLC plate. Plate was eluated in a vessel that contains the mobile phase chloroform: ethyl acetate (9:1), then see under UV_{254} nm. The spots marked with a needle, scraped, collected, diluted with chloroform: methanol (1:1) and then filtered, evaporated, until the isolates obtained in the form of crystals.

4.2. Phagocytosis assay

Latex beads was suspensed in PBS so that concentrations obtained 2.5 $x10^7$/ml. Medium taken so stay macrophages in coverslips. At each well was added 1ml of test material in complete RPMI medium in triplo (3 coverslips) and then incubated. After 4 hours incubation, suspension of latex beads added to PBS, where latex beads at a density of 400 mL latex beads in 2.5 $x10^7$/ml, then incubated in a CO_2 incubator for 60 minutes. Media collected, dried, painted with 20% Giemsa for 30 minutes, then washed with distilled water thoroughly removed from the culture wells and dried at room temperature (Leich et al., 1986; Wahyuniari, 2006, Derre and Isberg, 2004), each at 2 field of view for each coverslip.

4.3. Assay for nitrite determination by Griess reaction

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 (Cheng et al., 2008)

4.4. Lymphocytes proliferation assay with MTT Assay.

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% CO_2 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 (Wahyuniari, 2006).

5. Data Analysis :

Phagocytostic activity was measured by the latex-bead phagocytosis index (PI), the phagocytosis percentage (PP), and the phagocytosis efficiency (PE) (Sanchez et al., 2008). Expressed values are mean SD using one way anova, Tukey test, and Pearson correlation.

RESULT AND DISCUSSION

1. Isolation of compounds

Isolation of compound 1 (code Pc-1) and compound 2 (Pc-2 code) from 2.12 grams of red betel leaf methanolic extract produced 12.0 mg of Pc-1 and Pc-2 12.1 mg pure in TLC. Compound Pc-1 and Pc-2 is colored purple isolated on UV 254, UV invisible at 366, brown colour on detection with cerium sulfate as well as having Rf 0.25 at the price movement of chloroform: ethyl acetate (9:1), Pc-1 having Rf 0.6 and Rf Pc-2 has a 0.25; the mobile phase hexane: acetone (3:1), Pc-1 had Rf 0.5 and Pc-2 has Rf 0.19; the mobile phase hexane: ethyl acetate (3:1). Pc-1 had Rf 0.9 and Rf Pc-2 has a 0.8. Detection by TLC on Pc-1 and Pc-2 using 3 mobile phase systems showed a single spot on their TLC chromatogram. Compound Pc-1 and Pc-2 isolated from methanolic extract of red betel leaves is the same with Kustiawan's compound 1 and 2.

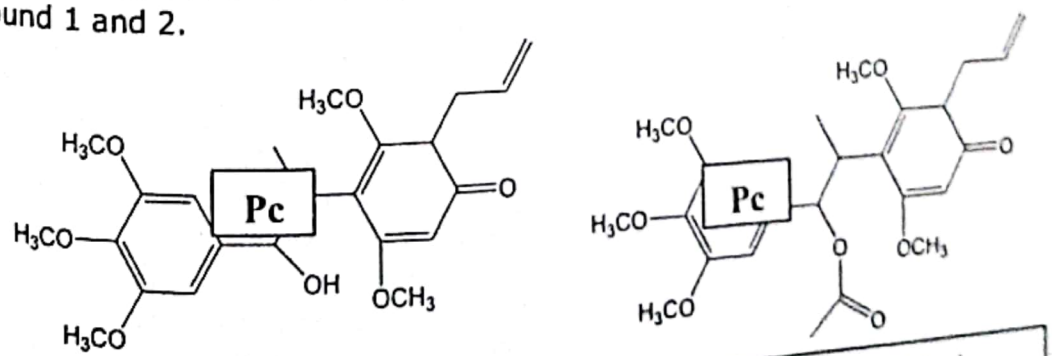

Figure 1. The two compounds (Pc-1 and Pc-2) from red betel (*Piper crocatum* Ruiz & Pav.)

2. Phagocytic activity

Both compounds were isolated from the leaves of red betel (*P. crocatum* Ruiz & Pav.) able to increase the phagocytic activity and capacity of peritoneal macrophages of mice infected with *L. monocytogenes*. Figure 2 shows the activity of

macrophages in phagocytic latex beads, whereas figure 3 shows the capacity of macrophages. The phagocytosis efficiency which is the ratio between the capacity of the activity is shown in figure 4. 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 both on 10th day and 21th day. At a dose of 5 mg/kgBW both Pc-1 and pc-2 a showed significant difference on 21th day, it means that 5 mg/kgBW is the minimal dose of Pc-1 and Pc-2 to increase activity and capacity of macrophage. The positive control group treated wih product containing 250 mg of Echinacea extract, the ability of echinacea extract in enhancing phagocytosis has been reported by Bauer (1999). On the 10th day, treated with 5 mg/kgBW and 2.5 mg/kgBW both Pc-1 and Pc-2 showed no significant differences for the three control groups. 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 on the 21th day, but not significant at the 10th day on phagocytic parameters percent. At a dose of 10 mg/kgBW Pc-2 macrophage phagocytic capacity is greater than Pc-1. For phagocytosis efficiency parameter, Pc-2 at a dose of 5 mg/kgBW or 10 mg/kgBW showed a significant difference on the 21th day, whereas Pc-1 only at a dose of 10 mg kgBW. Pearson correlation analysis showed that there is a positive close significant correlation between phagocytic percentage and phagocytic indeks (P>0.01). In this researh 2.12 g *P. crocatum* Ruiz & Pav. methanolic extract contains 12,0 mg Pc-1 and 12.1 mg Pc-2, therefore the dose of 5mg/kgBW Pc-2 equal to 876mg/kgBW extract while the dose of 5mg/kgBW Pc-1 equal to 883mg/kgBW extract. At the dose of 2.5 mg/kgBW both Pc-1 and Pc-2 showed no significant difference compare to the control group, it dose equal to 438mg/kgBW Pc2 and 442 mg/kgBW Pc1. Since Apriyanto (2011) and Indriyani (2011) reported that extract of *P. crocatum* Ruiz & Pav. at dose of 100mg/kgBW and 300 mg/kgBW increase phagocytic activity, it indicated that *in vivo* phagocytic assay showed single compound less active than extract.

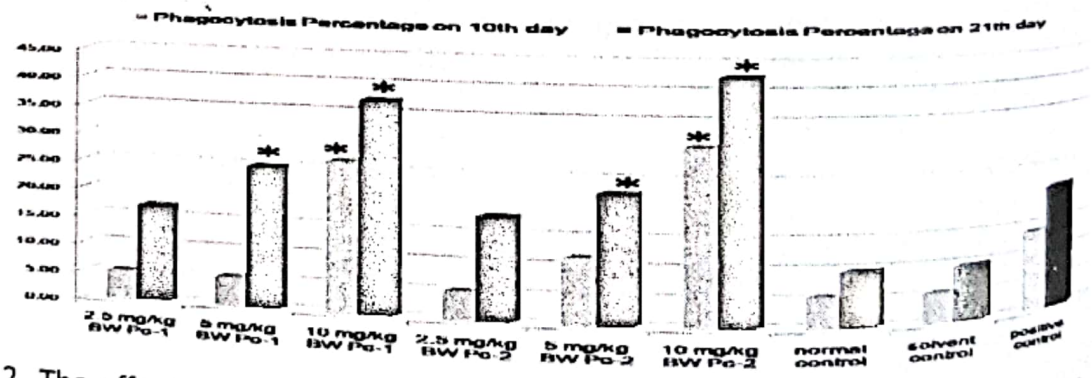

Figure 2. The effect of Pc-1 and Pc-2 on macrophage percentage phagocytosis on 10th day and 21th day. Values are the mean±SD of three replicates, * indicates statistically significant differences (P<0.05) vs normal control and solvent control.

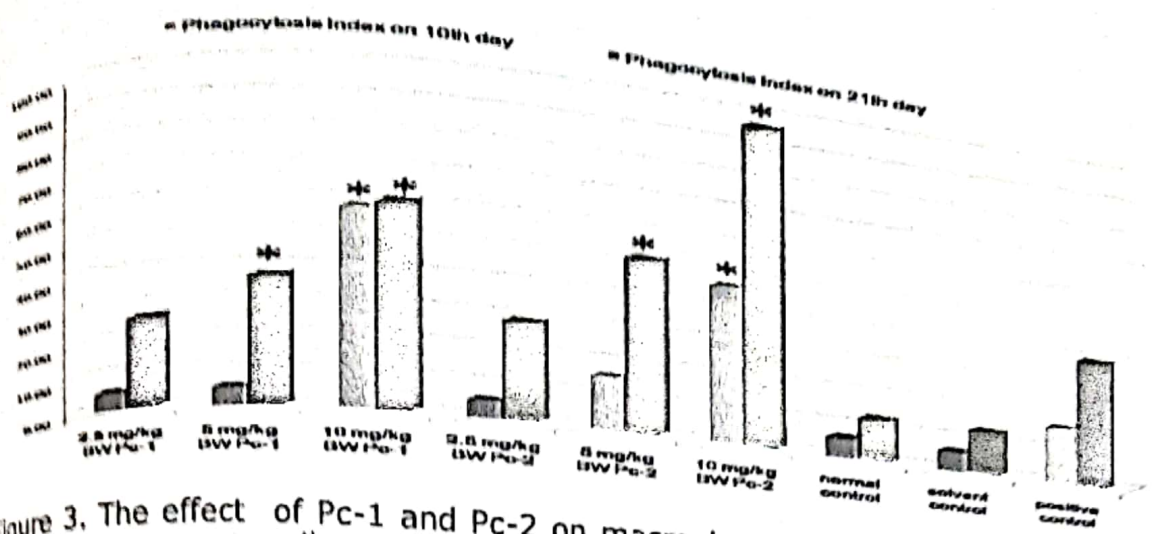

Figure 3. The effect of Pc-1 and Pc-2 on macrophage index phagocytosis on 10th day and 21th day. Values are the mean±SD of three replicates, * indicates statistically significant differences (P<0.05) vs normal control and solvent control

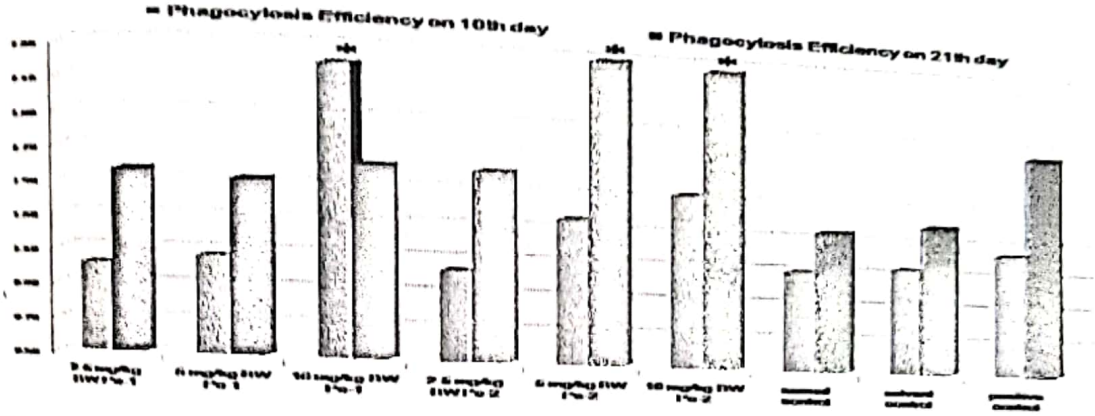

Figure 4. The effect of Pc-1 and Pc-2 on macrophage efficiency phagocytosis on 10th day and 21th day. Values are the mean±SD of three replicates, * indicates statistically significant differences (P<0.05) vs normal control and solvent control.

3. Nitrite production

Treatment with compound code Pc-1 and Pc-2 at a dose of 5 or 10 mg/kgBW showed a significant difference in nitrite production compare to treatment with normal control, solvent control and positive control on 10th day and 21th day, whereas at a dose of 2.5 mg/kgBW showed significant differences to the three control groups on the 21th day.

4. Lymphocytes proliferations

Lymphocyte proliferation assay showed no significant difference among Pc-1 and Pc-2, respectively at doses of 2.5, 5, and 10 mg/kgBW compare to solvent control and medium control group (P<0.05). There was no effect of both Pc-1 and Pc-2 on the lymphocytes proliferation of mice BALB/c, Apriyanto (2011) and Indriyani (2011) also reported that extract of *Piper crocatum* Ruiz & Pav. did'n effect on lymphocyte proliferation. Figure 5 showed that increases of doses tend to reduce the lymphocytes proliferation, it seem that *P. 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 *P. crocatum* Ruiz & Pav.; gave Immunosupressan on cellular and humoral response, moreover

Yuristiyani (2012) found that *in vitro* assay showed it suppres the lymphocytes proliferation.

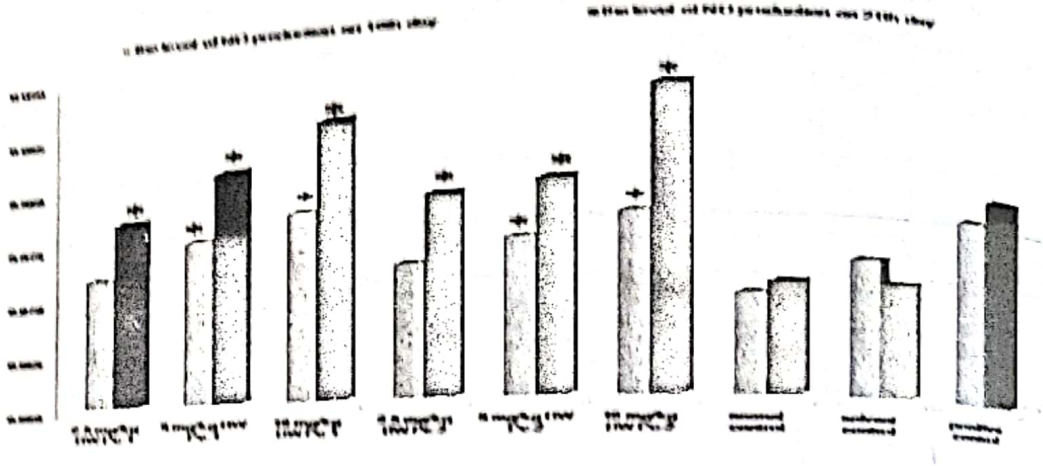

Figure 5. The effect of Pc-1 and Pc-2 on NO production on 10th day and 21th. Values are the mean±SD of three replicates, * indicates statistically significant differences (P<0.05) vs normal control and solvent control.

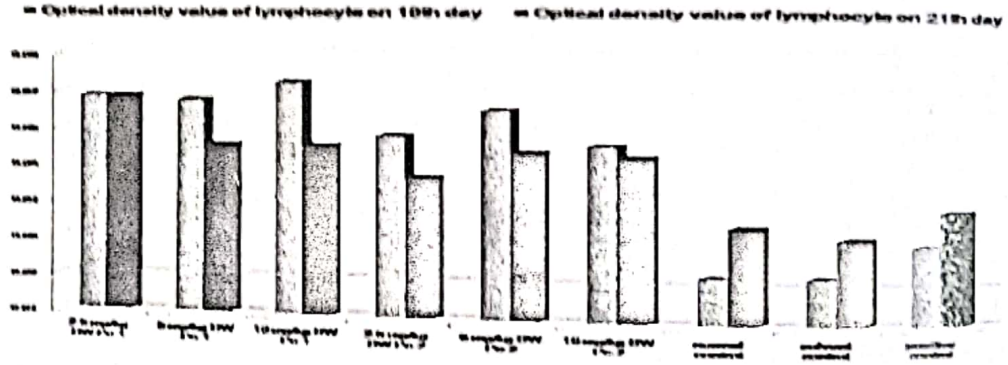

Figure 6. The effect of Pc-1 and Pc-2 on lymphocytes proliferation on 10th day and 21th day. Values are the mean±SD of three replicates, Pc-1 and Pc-2 do not indicates statistically significant differences (P<0.05) vs normal control and solvent control.

CONCLUSION

Both compound 1 (Pc-1) and compound 2 (Pc-2) from *Piper crocatum* Ruiz & Pav. increase the activity and capacity of macrophage. At in vivo dose of 5 and 10 mg/kgBW, they had significantly enhanced phagocytic activity compare to control group, and no significant difference between Pc-1 and Pc-2 (P<0.05). At dose of 2.5, 5, and 10 mg/kgBW, both Pc-1 and Pc-2 increase nitric oxide production, but didn't effect on lymphocyte proliferation.

REFERENCES

Apriyanto, S., 2011, 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*, Fakultas Farmasi Universitas Gadjah Mada, Yogyakarta.

Bauer R. Chemistry, Analysis and Immunological Investigations of Echinacea phytopharmaceuticals, In: Wagner H, editor. *Immunomodulatory Agents from Plants*. Basel, Switzerland: Birkhauser Verlag,1999. p. 41-89.

Bafna A.R. dan Misrha S.H. 2004. Imunomodulatory activity of methanol extracts of flower-heads of *Sphaeranthus indicus* Linn. *Ars Pharm*. 45 (3): 281-291.

Buhner, S.H., 1999. *Herbal Antibiotics, Natural Alternatives for Treating Drug-Resistant Bacteria*, Storey Publishing, North Adams.

Coll, J.C. and Bowden B.F., 1986. The Apllication of Vacuum Liquid Chromatography to The Separation of Terpene Mixtures. *J Nat Prod*. 49: 934-936.

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.

Cheng A., Wan, F., Wang, J, jin Z., and Xu X., 2008. Macrophage Immunomodulatory activity of polysaccharides isolated from *Glycyrrhiza uralensis* fish. *International Immunopharmacology*, 8:43-50.

Indriyani, L.W., 2011. 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*, Fakultas Farmasi Universitas Gadjah Mada, Yogyakarta.

Kanjwani, D.G., Marathe, T.P., Chipunkar, S.V., fan Sathaye, S.S., 2008, Evaluation of Immunomodulatory Activity of Methanolic Extract of Piper betel, *Scand J of Imm*, 67, 589-593.

Kustiawan, P.M., Wahyuono, S., dan Yuswanto, A., 2012. Isolasi dan Identifikasi Senyawa Imunostimulan Non Spesifik In Vitro dari Daun Sirih Merah (Piper crocatum Ruiz & Pav.). *Thesis*. Program Pascasarjana Fakultas Farmasi UGM

Leijh PCJ, Furth RV,Van Zwet TL. In Vitro Determination of Phagocytsis and Intracellular Killing by Polymorphonuclear and Mononuclear Phagocytes. dalam *Cellular Immnunology* (Weir, D.M., ed.). Blackwell Scientific Publications. Fourth Ed. Oxford. 1986. P. 46.3-46.6.

Sanchez S, Paredes SD, Sanchez CL, Barriga C, Reiter RJ, Rodriquez AB. 2008. Tryptophan Administration in rats Enhances Phagocytic Function and Reduces Oxidative Metabolism. *Neuro Endocrinol Lett*. 29(6):1026-1032.

Wagner H, Kraus S, and Jurcic K., 1999. Search for Potent Immunostimulating Agents from Plants and Other Natural Sources, In: Wagner H, editor. *Immunomodulatory Agents from Plants*. Basel, Switzerland: Birkhauser Verlag, p. 1-40.

Wahyuniari IAI.2006. Pengaruh Pemberian Minyak Buah Merah (*Pandanus conoideus* Lamp) pada Respon Imun Seluler Setelah Infeksi Listeria monocytogenes. *Tesis*. Program Studi Ilmu Kedokteran Dasar dan Biomedis, Sekolah Pascasarjana Universitas Gadjah Mada.Yogyakarta. p. 24-26.

Yuristiyani, D.D., 2012. 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*, Program Studi Farmasi, Fakultas Farmasi, Universitas Gadjah Mada, Yogyakarta.

In vivo immunomodulatory effect of two compounds isolated from red betel (*Piper crocatum* Ruiz & Pav.) on BABL/c mice infected with *Listeria monocytogenes*

Yustina Sri Hartini*, Subagus Wahyuono, Sitarina Widyarini***, Ag. Yuswanto****

*Faculty of Pharmacy Sanata Dharma University, **Faculty of Pharmacy Gadjah Mada University, ***Faculty of Veterinary Medicine Gadjah Mada University

Corresponding author: yustinahartini@usd.ac.id

As related research applications as an activator immunostimulatory immune system results are inconclusive, therefore required new immunostimulatory search of new source. Empirically red betel (*Piper crocatum* Ruiz & Pav) leaf is widely used as a traditional medicine to treat a variety of diseases, many plants used as traditional medicines reported to have immunomodulatory activity. Immunomodulatory activity study on Balb/c mice with *Listeria monocytogenes* infection of 2 compounds (code Pc-1 and Pc-2) isolated from the methanolic extract of red betel leaf have been done. The assessment of immunomodulatory activity were carried out by macrophage phagocytic assay, nitric oxide assay, and lymphocytes proliferation assay. Phagocytic activity assay showed that both Pc-1 and Pc-2 increases the activity and capacity of macrophage. At *in vivo* dose of 5 and 10 mg/kgBW, they had significantly enhanced phagocytic activity compare to control group, and no significant difference between Pc-1 and Pc-2 ($P<0.05$). Nitric oxide assay at dose of 2.5, 5, and 10 mg/kgBW of both Pc-1 and Pc-2 showed significantly difference compare to control group, but on lymphocyte proliferation assay they showed no significant difference ($P<0.05$).

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

INTRODUCTION

The immune system is the first line of defense of the human body, protecting it from disease and treat it if it occurs (Buhner, 1999). The human body continuously exposure by a variety of factors that result in lower immune function and increasing immunosuppression system. Research related to the

application as an activator immunostimulatory immune system results are inconclusive and need a new immunostimulatory search of new sources (Wagner *et al.*, 1999). Empirically red betel leaf is widely used as a traditional medicine to treat a variety of diseases, many plants used as traditional medicines reported to have immunostimulatory activity (Bafna and Misrha, 2004). Red betel (*Piper crocatum* Ruiz & Pav.) is used as a medicinal plant for various diseases. Kustiawan (2012) isolated 2 compounds of the methanolic extract of red betel leaf. The compound 1 (Pc-1) is 2-allyl-4-(1'-hydroxy-1'-(3",4",5"-trimethoxyphenyl) propan-2'-yl) -3,5-dimethoxycyclohexa-3, 5-dienone and compound 2 (Pc-2) is 2-allyl-4-(1'-acetyl-1'-(3",4",5"-trimethoxyphenyl) propan-2'-yl) -3,5-dimethoxycyclohexa- 3,5-dienone, and *in vitro* test of the two compounds showed immunostimulatory activity. According to Wagner *et al*. (1999), *in vitro* assay results do not always correlate with *in vivo* tests, it is necessary to confirm the *in vivo* test. This study reports the results of immunomodulatory effects *in vivo* test ie: macrophage phagocytosis, nitric oxide production, and lymphocyte proliferation mice treated with Pc-1 and Pc-2.

MATERIALS AND METHODS

1. Plant material :

The fresh leaves of red betel were collected from Balai Besar Penelitian dan Pengembangan Tanaman Obat dan Obat Tradisional, Central of Java, Indonesia on May 2010. Plant species was determined at department of

Biology Pharmacy Faculty of Pharmacy, Gadjah Mada University (GMU), Yogyakarta, Indonesia.

2. Animals and microorganisms:

Male Balb/c mice 8 weeks old weighing about 20-25 g obtained from Pharmacology Laboratory while *Listeria monocytogenes* obtained from Microbiology Laboratory, Faculty of Medicine GMU were used for the experiments. All procedures were approved by The Ethical Clearance Commision for pra-clinically research of Laboratorium Penelitian dan Pengujian Terpadu GMU. Balb/c mice were divided into nine groups. Group A, received 2.5 mg/kgBW Pc-1, Group B, received 5 mg/kgBW Pc-1, Group C, received 10 mg/kgBW Pc-1, Group D, received 2.5 mg/kgBW Pc-2, Group E, received 5 mg/kgBW Pc-2, Group F, received 10 mg/kgBW Pc-2, per oral for 14 days, Group G, didn't received drugs as normal control, Group H, received 0.75 ml of 1% sodium carboxy methyl cellulose per oral as solvent control, and Group I, received 100 mg/kgBW Produk-X® per oral as positive control. On 15^{th} day (=day 0) 0.2 ml *L. monocytogenes* containing $5x10^3$ cfu/ml are injected *intraperioneally* (ip) to all mice. On day 10, three mice of each group were used to obtaine 10^{th} day data, while 3 mice letf of all group are injected ip again with 0.2 ml *L. monocytogenes*. These mice are sacrificed on day 21 to obtain 21^{th} day data.

3. Chemicals:

Medium *Rosewell Park Memorial Institute* (RPMI)-1640 (Sigma R-6504), *fetal bovine seru*m (FBS) (Gibco BRL 10082147), 1mM sodium bicarbonate ($NaHCO_3$) (Sigma S-5761), mM L-Glutamin 100, µg penisilin, dan 0.5 mg streptomisin (Gibco 15140-122), fungison (Gibco 15295-017), *Phosphate-buffered saline (PBS),* chloroform (Merck K 23788209709), methanol (Merck K 2538245), *latex beads* (Sigma LB-30) diameter 0,3µm, Giemsa 10%, *tryphan blue,* Product-X® (kaplet Imboost Force PT Kimia Farma).

4. Methods:

4.1. Compounds isolation.

Red betel leaf methanolic extract was fractionated by the method of vacuum liquid chromatography (VLC) (Coll and Bowden, 1986) successively with n-hexane, chloroform, ethyl acetate, and methanol so that the final fraction obtained 5. Isolated compounds (Pc-1 and Pc-2) are compounds with a purple marker spots that reduce UV_{254}, no color at UV_{366} nm, and brown on the detection of cerium sulfate, with a mobile phase of chloroform: ethyl acetate (9:1). The Pc-1 (Rf 0:25) and Pc-2 (Rf 0.7) contained in fraction III and fraction IV results VLC separation of methanolic extract of red betel leaf. Fraction was dissolved in chloroform: methanol (1:1) and then spotted on preparative TLC plate. Plate was eluated in a vessel that contains the mobile phase chloroform: ethyl acetate (9:1), then see under UV_{254} nm. The spots marked with a needle, scraped, collected, diluted with chloroform: methanol (1:1) and then filtered, evaporated, until the isolates obtained in the form of crystals.

4.2. Phagocytosis assay. Latex beads was suspensed in PBS so that concentrations obtained 2.5 x10^7/ml. Medium taken so stay macrophages in coverslips. At each well was added 1ml of test material in complete RPMI medium in triplo (3 coverslips) and then incubated. After 4 hours incubation, suspension of latex beads added to PBS, where latex beads at a density of 400 mL latex beads in 2.5 x10^7/ml, then incubated in a CO_2 incubator for 60 minutes. Media collected, dried, painted with 20% Giemsa for 30 minutes, then washed with distilled water thoroughly removed from the culture wells and dried at room temperature (Leich *et al*., 1986; Wahyuniari, 2006, Derre and Isberg, 2004), each at 2 field of view for each coverslip.

4.3. Assay for nitrite determination by Griess reaction

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 (Cheng *et al*., 2008)

4.4. Lymphocytes proliferation assay with MTT Assay. 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% CO_2 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 (Wahyuniari, 2006).

5. Data Analysis :

Phagocytostic activity was measured by the latex-bead phagocytosis index (PI), the phagocytosis percentage (PP), and the phagocytosis efficiency (PE) (Sanchez *et al.,* 2008). Expressed values are mean SD using one way anova, Tukey test, and Pearson correlation.

RESULT AND DISCUSSION

1. Isolation of compounds

Isolation of compound 1 (code Pc-1) and compound 2 (Pc-2 code) from 2.12 grams of red betel leaf methanolic extract produced 12.0 mg of Pc-1 and Pc-2 12.1 mg pure in TLC. Compound Pc-1 and Pc-2 is colored purple isolated on UV 254, UV invisible at 366, brown colour on detection with cerium sulfate as well as having Rf 0.25 at the price movement of chloroform: ethyl acetate (9:1), Pc-1 having Rf 0.6 and Rf Pc-2 has a 0.25; the mobile phase hexane: acetone (3:1), Pc-1 had Rf 0.5 and Pc-2 has Rf 0.19; the mobile phase hexane: ethyl acetate (3:1). Pc-1 had Rf 0.9 and Rf Pc-2 has a 0.8. Detection by TLC on Pc-1 and Pc-2 using 3 mobile phase systems showed a single spot on their TLC chromatogram. Compound Pc-1 and Pc-2 isolated from methanolic extract of red betel leaves is the same with Kustiawan’s compound 1 and 2.

2. Phagocytic activity

Both compounds were isolated from the leaves of red betel (*P. crocatum* Ruiz & Pav.) able to increase the phagocytic activity and capacity of peritoneal macrophages of mice infected with *L. monocytogenes*. Figure 2 shows the activity of macrophages in phagocytic latex beads, whereas figure 3 shows the capacity of macrophages. The phagocytosis efficiency which is the ratio between the capacity of the activity is shown in figure 4. 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 both on 10^{th} day and 21^{th} day. At a dose of 5 mg/kgBW both Pc-1 and pc-2 a showed significant difference on 21^{th} day, it means that 5 mg/kgBW is the minimal dose of Pc-1 and Pc-2 to increase activity and capacity of macrophage. The positive control group treated wih product containing 250 mg of Echinacea extract, the ability of echinacea extract in enhancing phagocytosis has been reported by Bauer (1999). On the 10^{th} day, treated with 5 mg/kgBW and 2.5 mg/kgBW both Pc-1 and Pc-2 showed no significant differences for the three control groups. 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 on the 21^{th} day, but not significant at the 10^{th} day or phagocytic parameters percent. At a dose of 10 mg/kgBW Pc-2 macrophage phagocytic capacity is greater than Pc-1. For phagocytosis efficiency parameter, Pc-2 at a dose of 5 mg/kgBW or 10 mg/kgBW showed a significant

difference on the 21th day, whereas Pc-1 only at a dose of 10 mg kgBW. Pearson correlation analysis showed that there is a positive close significant correlation between phagocytic percentage and phagocytic indeks (P>0.01). In this researh 2.12 g *P. crocatum* Ruiz & Pav. methanolic extract contains 12,0 mg Pc-1 and 12.1 mg Pc-2, therefore the dose of 5mg/kgBW Pc-2 equal to 876mg/kgBW extract while the dose of 5mg/kgBW Pc-1 equal to 883mg/kgBW extract. At the dose of 2.5 mg/kgBW both Pc-1 and Pc-2 showed no significant difference compare to the control group, it dose equal to 438mg/kgBW Pc2 and 442 mg/kgBW Pc1. Since Apriyanto (2011) and Indriyani (2011) reported that extract of *P. crocatum* Ruiz & Pav. at dose of 100mg/kgBW and 300 mg/kgBW increase phagocytic activity, it indicated that *in vivo* phagocytic assay showed single compound less active than extract.

3. Nitrite production

Treatment with compound code Pc-1 and Pc-2 at a dose of 5 or 10 mg/kgBW showed a significant difference in nitrite production compare to treatment with normal control, solvent control and positive control on 10th day and 21th day, whereas at a dose of 2.5 mg/kgBW showed significant differences to the three control groups on the 21th day.

4. Lymphocytes proliferations

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

and Pc-2 on the lymphocytes proliferation of mice BALB/c, Apriyanto (2011) and Indriyani (2011) also reported that extract of *Piper crocatum* Ruiz & Pav. did'n effect on lymphocyte proliferation. Figure 5 showed that increases of doses tend to reduce the lymphocytes proliferation, it seem that *P. 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 *P. crocatum* Ruiz & Pav.; gave immunosupressan on cellular and humoral response, moreover Yuristiyani (2012) found that *in vitro* assay showed it suppres the lymphocytes proliferation.

CONCLUSION

Both compound 1 (Pc-1) and compound 2 (Pc-2) from *Piper crocatum* Ruiz & Pav. increase the activity and capacity of macrophage. At *in vivo* dose of 5 and 10 mg/kgBW, they had significantly enhanced phagocytic activity compare to control group, and no significant difference between Pc-1 and Pc-2 ($P<0.05$). At dose of 2.5, 5, and 10 mg/kgBW, both Pc-1 and Pc-2 increase nitric oxide production, but didn't effect on lymphocyte proliferation.

REFERENCES

Apriyanto, S., 2011, 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*, Fakultas Farmasi Universitas Gadjah Mada, Yogyakarta.

Bauer R. Chemistry, Analysis and Immunological Investigations of Echinachea Phytopharmaceuticals, In: Wagner H, editor. *Immunomodulatory Agents from Plants.* Basel, Switzerland: Birkhauser Verlag,1999. p. 41-89.

Bafna A.R. dan Misrha S.H. 2004. Imunomodulatory activity of methanol extracts of flower-heads of *Sphaeranthus indicus* Linn. *Ars Phar*m. 45 (3): 281-291.

Buhner, S.H., 1999. *Herbal Antibiotics, Natural Alternatives for Treating Drug-Resistant Bacteria*, Storey Publishing, North Adams.

Coll, J.C. and Bowden B.F., 1986. The Apllication of Vacuum Liquid Chromatography to The Separation of Terpene Mixtures. *J Nat Prod.* 49: 934-936.

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.

Cheng A., Wan, F., Wang, J, jin Z., and Xu X., 2008. Macrophage immunomodulatory activity of polysaccharides isolated from *Glycyrrhiza uralensis* fish. *International Immunopharmacology,* 8:43-50.

Indriyani, L.W., 2011. 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*, Fakultas Farmasi Universitas Gadjah Mada, Yogyakarta.

Kanjwani, D.G., Marathe, T.P., Chipunkar, S.V., fan Sathaye, S.S., 2008, Evaluation of Immunomodulatory Activity of Methanolic Extract of Piper betel, *Scand J of Imm*, 67, 589-593.

Kustiawan, P.M., Wahyuono, S., dan Yuswanto, A., 2012. Isolasi dan Identifikasi Senyawa Imunostimulan Non Spesifik In Vitro dari Daun Sirih Merah (Piper crocatum Ruiz & Pav.). *Thesis.* Program Pascasarjana Fakultas Farmasi UGM

Leijh PCJ, Furth RV,Van Zwet TL. In Vitro Determination of Phagocytsis and Intracellular Killing by Polymorphonuclear and Mononuclear Phagocytes. dalam *Cellular Immnunology* (Weir, D.M., ed.). Blackwell Scientific Publications. Fourth Ed. Oxford. 1986. P. 46.3-46.6.

Sanchez S, Paredes SD, Sanchez CL, Barriga C, Reiter RJ, Rodriquez AB. 2008. Tryptophan Administration in rats Enhances Phagocytic Function and Reduces Oxidative Metabolism. *Neuro Endocrinol Lett*. 29(6):1026-1032.

Wagner H, Kraus S, and Jurcic K., 1999. Search for Potent Immunostimulating Agents from Plants and Other Natural Sources, In: Wagner H, editor. *Immunomodulatory Agents from Plants.* Basel, Switzerland: Birkhauser Verlag, p. 1-40.

Wahyuniari IAI.2006. Pengaruh Pemberian Minyak Buah Merah (*Pandanus conoideus* Lamp) pada Respon Imun Seluler Setelah Infeksi Listeria monocytogenes. *Tesis.* Program Studi Ilmu Kedokteran Dasar dan Biomedis, Sekolah Pascasarjana Universitas Gadjah Mada.Yogyakarta. p. 24-26.

Yuristiyani, D.D., 2012. 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*, Program Studi Farmasi, Fakultas Farmasi, Universitas Gadjah Mada, Yogyakarta.

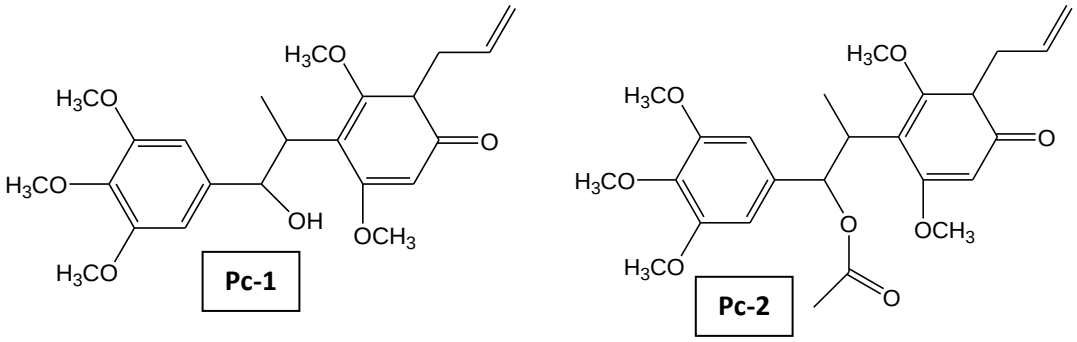

Figure 1. The two compounds (Pc-1 and Pc-2) from red betel (*Piper crocatum* Ruiz & Pav.)

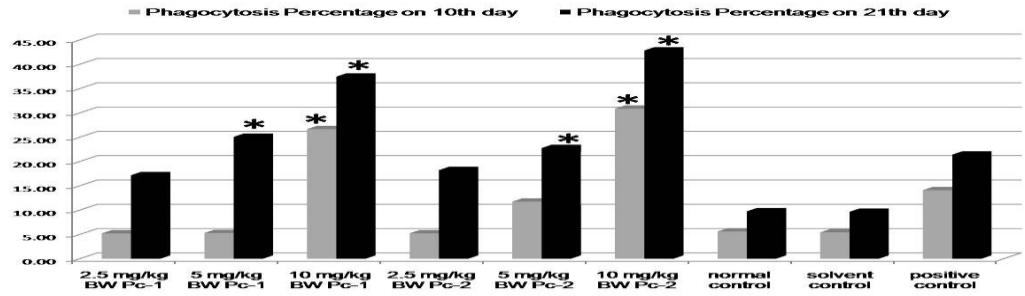

Figure 2. The effect of Pc-1 and Pc-2 on macrophage percentage phagocytosis on 10th day and 21th day. Values are the mean±SD of three replicates, * indicates statistically significant differences (P<0.05) vs normal control and solvent control.

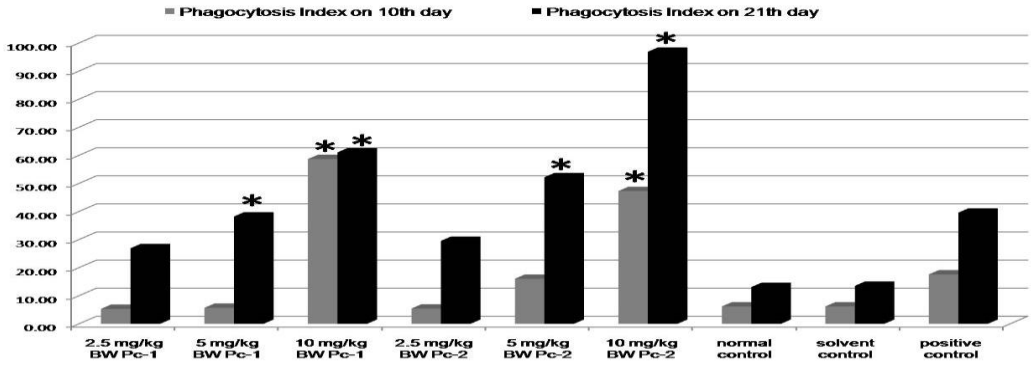

Figure 3. The effect of Pc-1 and Pc-2 on macrophage index phagocytosis on 10th day and 21th day. Values are the mean±SD of three replicates, * indicates statistically significant differences (P<0.05) vs normal control and solvent control

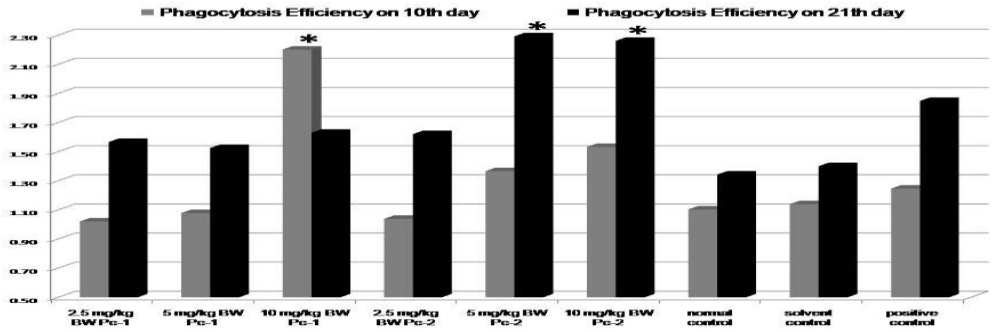

Figure 4. The effect of Pc-1 and Pc-2 on macrophage efficiency phagocytosis on 10th day and 21th day. Values are the mean±SD of three replicates, * indicates statistically significant differences (P<0.05) vs normal control and solvent control.

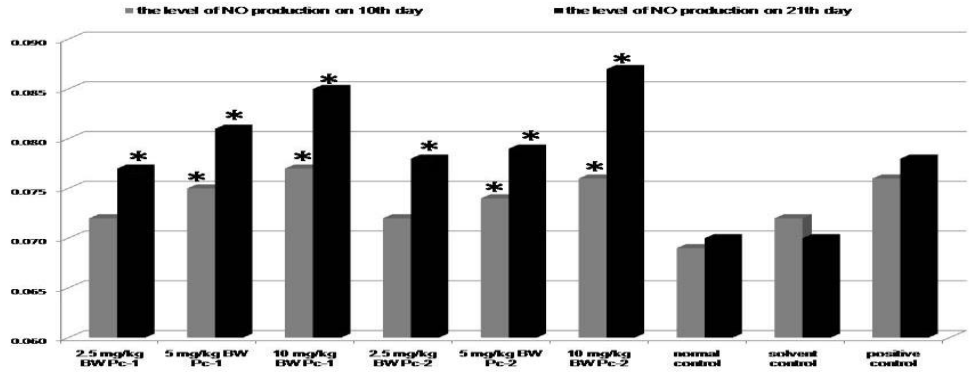

Figure 5. The effect of Pc-1 and Pc-2 on NO production on 10th day and 21th. Values are the mean±SD of three replicates, * indicates statistically significant differences (P<0.05) vs normal control and solvent control.

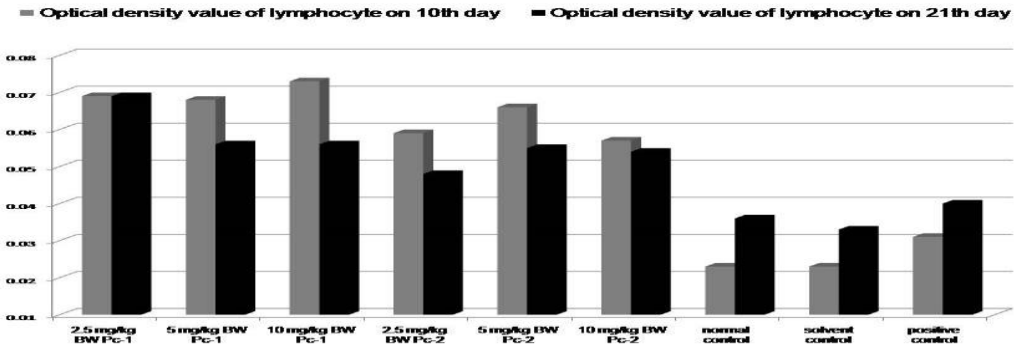

Figure 6. The effect of Pc-1 and Pc-2 on lymphocytes proliferation on 10^{th} day and 21^{th} day. Values are the mean±SD of three replicates, Pc-1 and Pc-2 do not indicates statistically significant differences ($P<0.05$) vs normal control and solvent control.