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

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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). Some of the limitations of existing imunostimulaton is short half-lives and the resulting immunity is only partial, and unstable, but it is quite high toxicity of compounds, especially in chronic use (Iwo, 2006). 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 an ornamental plant because its silvery shiny red leaves, lately people in Indonesia use as a medicinal plant for various diseases. Kustiawan (2012) isolated 2 compounds of the methanolic extract of red betel leaf. The compound 1 is 2-allyl-4-(1'-hydroxy-1'-(3 '', 4'', 5 ''-trimethoxyphenyl) propan-2'-yl) -3,5-dimethoxycyclohexa-3, 5-dienone and compound 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 compound 1 (code Pc-1) and compound 2 (code Pc-2).

MATERIALS AND METHODS

1. Plant material :

The fresh leaves of red betel (*Piper crocatum* Ruiz & Pav.) were collected from Balai Besar Penelitian dan Pengembangan Tanaman Obat dan Obat Tradisional (B2P2TO2T), Central of Java, Indonesia on May 2010. Plant species was determined at department of Biology Pharmacy Facultyy of Pharmacy, Gadjah Mada University, 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 Gadjah Mada University 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/kgBB Produk-X® per oral as positive control. On 15^{th} day (= day 0) 0.2 ml *Listeria monocytogenes* containing $5x10^3$ cfu/ml are

injected intraperioneally 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 intraperioneally again with 0.2 ml *Listeria monocytogenes* containing $5x10^3$ cfu/ml. 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 nm, no color at UV 366, 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 of fraction spot appears dry, plate was eluated in a vessel that contains the mobile phase chloroform: ethyl acetate (9:1) until the movement phase reaches 1 cm at the bottom end of the plate. The plates were removed from the vessel, then dry awaited diihat under UV254 nm, which dampen spots marked with a needle, and then scraped. Results scrapings were 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 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 (stick and went into the macrophage cell) is calculated by examined using a light microscope with a magnification of 400x (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% 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 (Wahyuniari, 2006).

5. Statistical Analysis :

Phagocytosis was Measured by the latex-bead phagocytosis index (PI), the phagocytosis percentage (PP), and the phagocytosis efficiency (PE) (Sanchez et al., 2008). The latex-bead phagocytosis index (PI), which is the number of latex beads ingested by 100 macrophages is the amount of latex that are eaten by macrophages 100 of 100 divided by the number of macrophages in the coverslip 3 times the number of macrophages in the latex is eaten 3 coverslip. Percent phagocytosis is the percent of cells that eating at least 1 latex that is the number of macrophages in 3 coverslip who eat at least 1 latex divided by the number of macrophages in 3 coverslip multiplied by 100%. Phagocytosis efficiency is the ratio between the index and the percent phagocytosis phagocytosis. 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, colored cokat 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 phase systems of motion showed a single spot on the TLC chromatogram third. Compound Pc-1 and Pc-2 isolated from methanolic extract of red betel leaves with isolates 1 and 2 were isolated by Kustiawan (2012), Pc-2 differs from Pc-1 for atom C1 'binding acetyl group. phenylpropanoid compounds from Piper kadsura.

2. Phagocytic activity

Both compounds were isolated from the leaves of red betel (*Piper crocatum* Ruiz & Pav.) able to increase the phagocytic activity and capacity of peritoneal macrophages of mice infected with *Listeria monocytogenes*. Figure 1 shows the activity of macrophages in phagocytic latex beads, whereas figure 2 shows the capacity of macrophages. The phagocytosis efficiency which is the ratio between the capacity of the activity is shown in figure 3. 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 after the mice were infected with *Listeria monocytogenes*. 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 percent phagocytosis and phagocytic index of macrophage groups of mice treated with 10 mg/kgBW Pc-1 and Pc-2 showed a significant different

compare to positive control on both on 10th day and 21th day after the mice were infected with *Listeria monocytogenes*. 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 after the mice infected with *Listeria monocytogenes* 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 after the mice were infected with *Listeria monocytogenes*, but not significant at the 10th day after the mice were infected with *Listeria monocytogenes* or phagocytic parameters percent. At a dose of 10 mg / kg 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 after the mice were infected with *Listeria monocytogenes*, whereas Pc-1 only at a dose of 10 mg kgBW. Pearson correlation analysis showed that there is a significant correlation between phagocytic percentage and phagocytic indeks (P>0.01), in this research there is a positive close correlation between macrophage activity and their capacity. In this researh 2.12 g *Piper 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 *Piper 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 10^{th} day and 21^{th} day after *Lysteria monocytogenes* infection, whereas at a dose of 2.5 mg/kgBW showed significant differences to the three control groups on the 21^{th} day after *Lysteria monocytogenes* infection.

4. Proliferation lymphocytes

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 4 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.

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.

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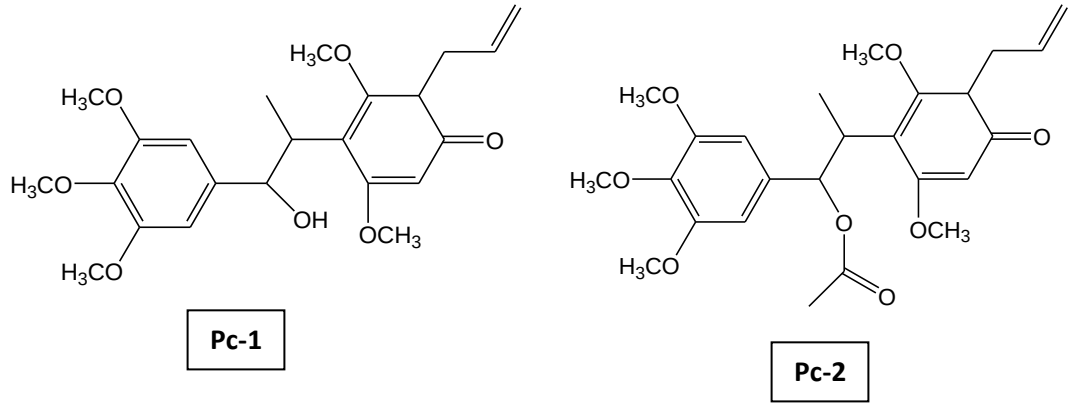

Figure 1. The two compounds (Pc-1 and Pc-2) from red betel (*Piper crocatum* Ruiz & Pav.)

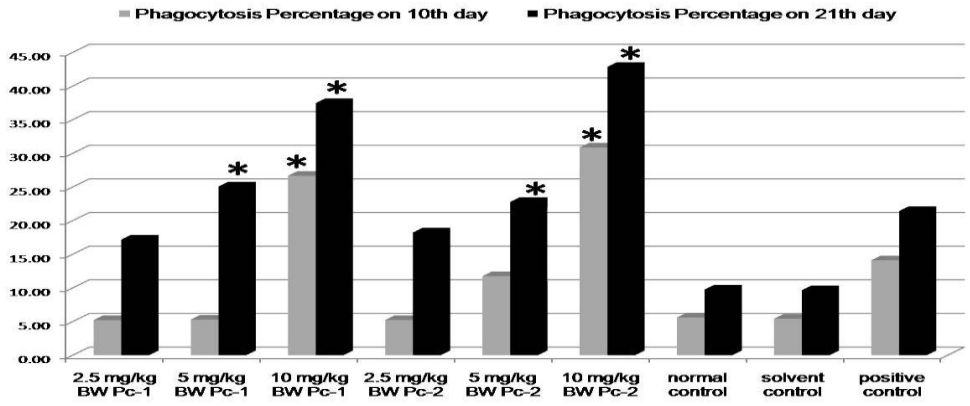

Figure 1. The effect of Pc-1 and Pc-2 on macrophage percentage phagocytosis on 10th day and 21th day after *Lysteria monocytogenes* infection on BALB/c mice. Values are the mean±SD of three replicates, * indicates statistically significant differences (P<0.05) vs normal control and solvent control (ANOVA)

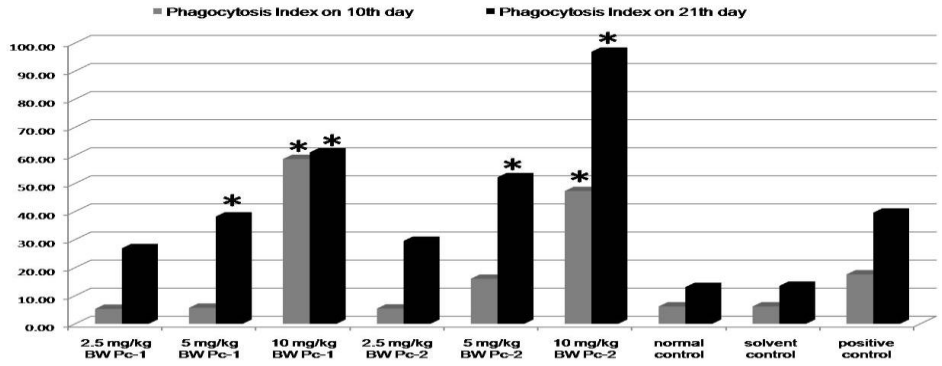

Figure 2. The effect of Pc-1 and Pc-2 on macrophage index phagocytosis on 10th day and 21th day after *Lysteria monocytogenes* infection on BALB/c mice. Values are the mean±SD of three replicates, * indicates statistically significant differences (P<0.05) vs normal control and solvent control (ANOVA)

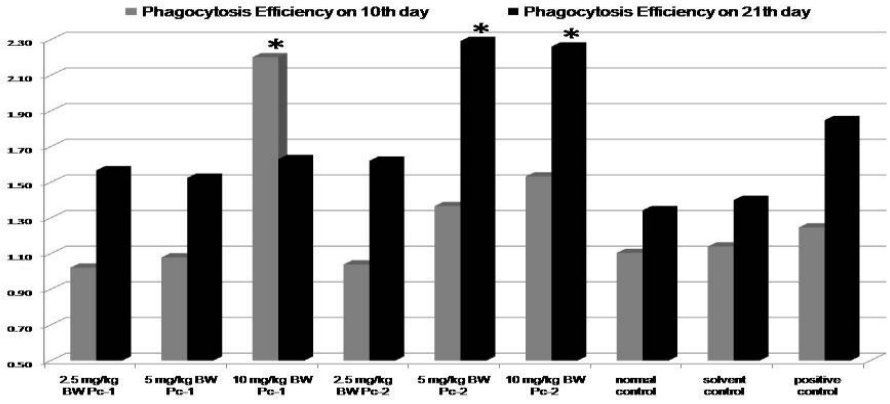

Figure3. The effect of Pc-1 and Pc-2 on macrophage efficiency phagocytosis on 10th day and 21th day after *Lysteria monocytogenes* infection on BALB/c mice. Values are the mean±SD of three replicates, * indicates statistically significant differences (P<0.05) vs normal control and solvent control (ANOVA)

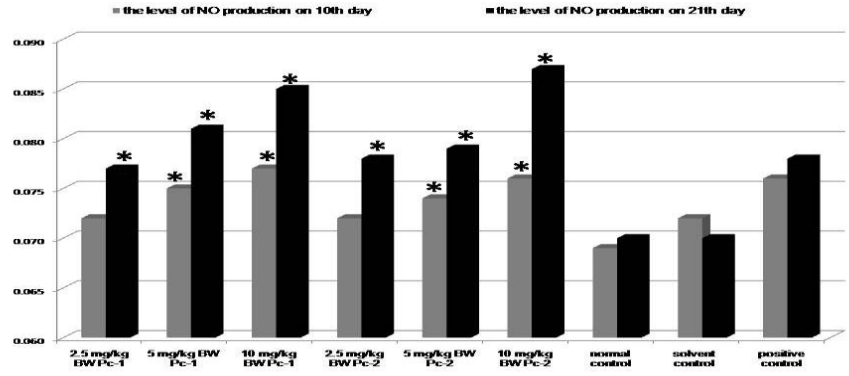

Figure 5. The effect of Pc-1 and Pc-2 on NO production on 10th day and 21th day after *Lysteria monocytogenes* infection on BALB/c mice. Values are the mean±SD of three replicates, * indicates statistically significant differences (P<0.05) vs normal control and solvent control (ANOVA)

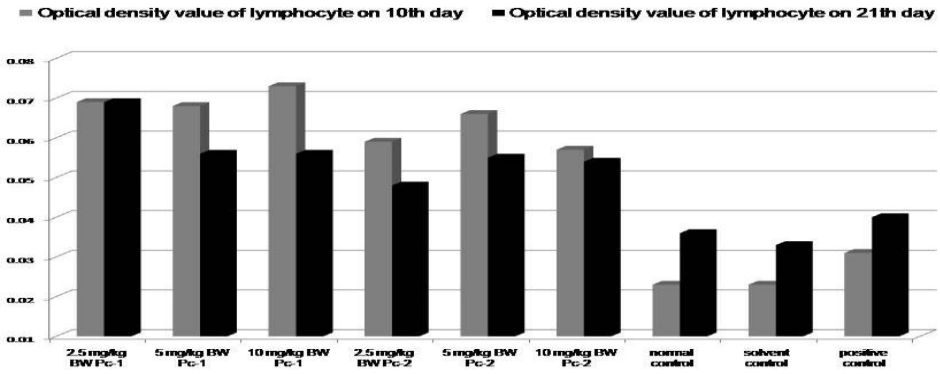

Figure 4. The effect of Pc-1 and Pc-2 on lymphocytes proliferation on 10th day and 21th day after *Lysteria monocytogenes* infection on BALB/c mice. Values are the mean±SD of three replicates, Pc-1 and Pc-2 do not indicates statistically significant differences (P<0.05) vs normal control and solvent control (ANOVA)