

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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Submission date: 18-Nov-2021 07:59AM (UTC+0700)

Submission ID: 1706093711

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Word count: 2918

Character count: 15368

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

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As related research applications as an activator immunostimulatory immune system results are inconclusive, ¹² therefore required new immunostimulatory ¹² arch 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 ² 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 ¹ 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 ²² 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

¹⁹
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 Misra, 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.

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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 Commission for pre-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 5×10^3 cfu/ml are injected *intraperitoneally* (ip) to all mice. On day 10, three mice of each group were used to obtain 10th day data, while 3 mice left 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 ⁶ *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), ⁶ *latex beads* (Sigma LB-30) diameter 0,3 μm , Giemsa 10%, *tryphan blue*, Product-X[®] (kaplet Imboost Force PT Kimia Farma).

4. Methods:

4.1. Compounds isolation.

¹ Red betel leaf methanolic extract was fractionated by ¹⁵ 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 ¹ 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 (R_f 0:25) and Pc-2 (R_f 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 suspended in PBS so that concentrations obtained $2.5 \times 10^7/\text{ml}$. 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 $2.5 \times 10^7/\text{ml}$, 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 $20\mu\text{M}$ concentration range up to $0.078 \mu\text{M}$. 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 phytohaemagglutinin (PHA) at a final concentration of 50 ug/ml, 10 ml/wells, and then incubated at 37°C . The 3-(4,5-dimethylthiazol-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. 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 were² isolated from the leaves of red betel (*P. crocatum* Ruiz & Pav.) able to increase²⁴ 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. Compounds¹ Pc-1 and Pc-2 showed the same pattern of effects on macrophage phagocytic activity and capacity.⁷ At a dose of 10 mg/kgBW both Pc-1 and pc-2¹ is able to increase the activity of macrophages and capacity both on 10th day and 21th day. At a dose of¹ 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 dose¹ of Pc-1 and Pc-2 to increase activity and capacity of macrophage. The positive control group treated with 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 showed¹ no significant differences for the three control groups.²³ 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 or phagocytic parameters percent.⁷ At a dose of 10 mg/kgBW Pc-2 macrophage phagocytic capacity is greater than Pc-1. For phagocytosis efficiency parameter,¹ 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 research 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.

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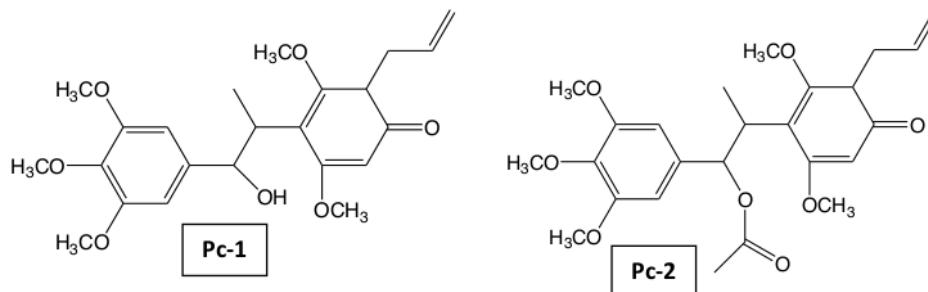


Figure 1. The two compounds (Pc-1 and Pc-2) from red betel (*Piper crocatum* Ruiz & Pav.)

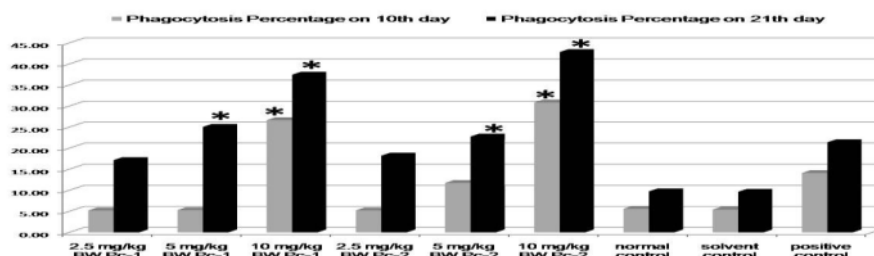


Figure 2. The effect of Pc-1 and Pc-2 on macrophage percent phagocytosis on 10th day and 21st day. Values are the mean±SD of three replicates, * indicates statistically significant differences (P<0.05) vs normal control and solvent control.

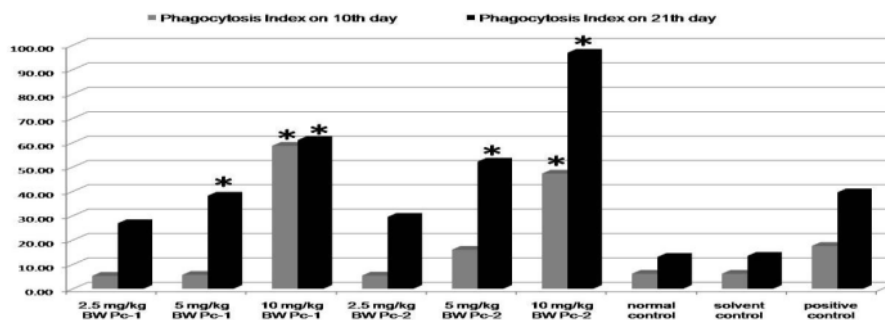
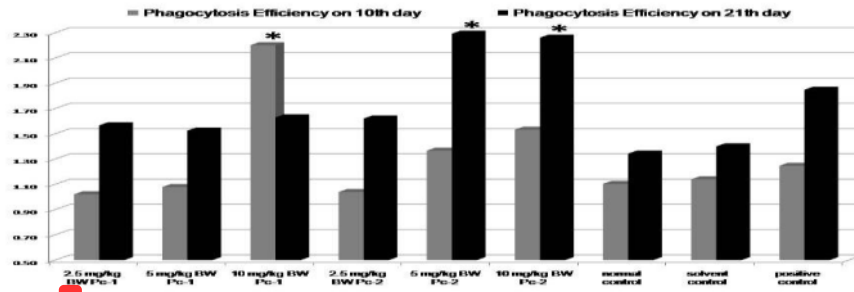
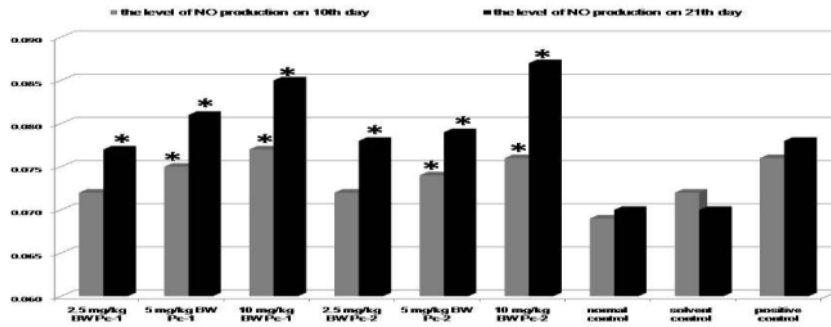


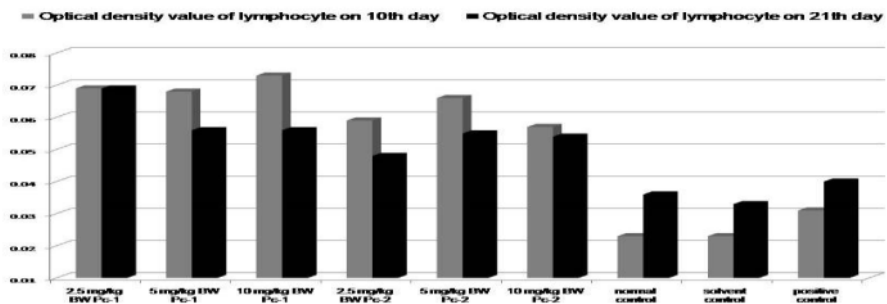
Figure 3. The effect of Pc-1 and Pc-2 on macrophage index phagocytosis on 10th day and 21st day. Values are the mean±SD of three replicates, * indicates statistically significant differences (P<0.05) vs normal control and solvent control



1 Figure 4. The effect of Pc-1 and Pc-2 on macrophage efficiency 5 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.



1 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.



1
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.

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