

TJPR



Tropical Journal of
Pharmaceutical Research

Vol 9 no.4
August 2010

Pharmacotherapy Group
<http://www.tjpr.org>

ISSN : 1596-3596
e-ISSN: 1596-9827

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Journal Identifiers

eISSN: 1596-9827

print ISSN: 1596-5996



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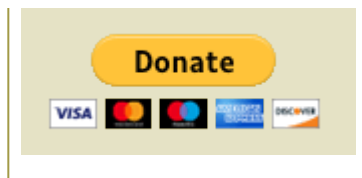
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Original Research Article

***In vivo* Immunomodulatory Effect and Histopathological Features of Mouse Liver and Kidney Treated with Neolignans Isolated from Red Betel (*Piper crocatum* Ruiz & Pav) Leaf**

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Received: 22 July 2014

Revised accepted: 16 September 2014

Abstract

Purpose: To investigate *in vivo* immunomodulatory effect and histopathological feature of mouse liver and kidney following treatment with 2 neolignans (Pc-1 and Pc-2) isolated from red betel (*Piper crocatum* Ruiz & Pav) leaf.

Methods: Balb/c mice immune response was induced with *Listeria monocytogenes*. Immunomodulatory effect was tested by using macrophage phagocytic, nitric oxide, and lymphocyte proliferation assays. The morphological features of liver and kidney were observed with light microscope and then compared with the liver and kidney of control group.

Results: At the dose of 5 and 10 mg/kg body weight, both Pc-1 and Pc-2 significantly increased the activity and the capacity of macrophages ($p < 0.05$). Both Pc-1 and Pc-2 significantly increased phagocytic activity of macrophage by 25% and 23%, respectively, and phagocytic index to 38 and 52, respectively at a dose of 5 mg/kg body weight. Increases in nitric oxide production due to Pc-1 and Pc-2 (at doses of 2.5, 5, and, 10 mg/kg body weight) were also observed although no lymphocyte proliferation effect was observed. Histopathological examination of liver and kidney of mice given Pc-1 demonstrated normal features. On the other hand, hydropic degeneration and liver necrosis were seen in mice given Pc-2 treatment. Based on this result and the structure similarity of the two compounds (Pc-1 and Pc-2), an interesting presumption was made that the -OH functional group (Pc-2) was responsible for the toxicity that caused liver damage.

Conclusion: The two neolignans (Pc-1 and Pc-2) isolated from the leaves of *P. crocatum* Ruiz & Pav. are capable of increasing macrophage phagocytosis as well as nitric oxide production but not lymphocyte proliferation. Histopathological features of liver given Pc-2 demonstrate hydropic degeneration and necrosis, possibly due to the -OH group on Pc-2.

Keywords: *Piper crocatum* Ruiz & Pav, Immunomodulatory, Liver necrosis, Kidney, Hydropic degeneration, Macrophage phagocytosis

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INTRODUCTION

Research related to the application of immunostimulants in the immune system has not lead to the conclusion that firm and need a new immunostimulatory and search for new sources of novel immunostimulatory. Many plants that are used as traditional medicines are reported to have immunostimulatory activity [1]. Nearly 1000 species of the genus *Piper* have been used by humans for traditional medicine [2]. Red betel (*Piper crocatum* Ruiz & Pav) is a species of genus *Piper* which have red silvery leaves. In Indonesia, red betel is used as a medicinal plant for treating various diseases, the methanolic extract was reported to have antiproliferative effect on human breast (T47D) cells [3].

Phytochemical investigation of *Piper* species has led to the isolation of a large number of physiologically active compounds including neolignans [2]. Kustiawan [4], demonstrated that neolignan from red betel has an effect on macrophage phagocytic activity in vitro. In this study, we report immunomodulatory effect of two neolignans (crocatidin and deacetyl crocatidin) isolated from red betel in Balb/c mice ie: macrophage phagocytic, nitric oxide production, and lymphocyte proliferation test. Histopathological features of the liver and kidney were also observed.

EXPERIMENTAL

Plant material

The fresh leaves of red betel (*Piper crocatum* Ruiz & Pav.) were collected from Tawangmangu Central of Java, Indonesia in May 2010. Plant species was authenticated by Wahyono of the Department of Biology, Faculty of Pharmacy, Gadjah Mada University, Yogyakarta, Indonesia and a voucher specimen (no. BF/284/Ident/Det/VIII/2011) was deposited in herbarium unit at The Faculty of Pharmacy, Sanata Dharma University, Indonesia.

Animals

Male Balb/c mice, aged 8 weeks were used in this study. Mice were divided into nine groups of six. Groups A, B, C were given crocatidin at the dose of 2.5, 5 mg/kg, and 10 mg/kg body weight, respectively. Groups D, E and F were given deacetyl crocatidin at the dose of 2.5, 5, and 10 mg/kg body weight, respectively. Both crocatidin and deacetyl crocatidin were orally administered once daily for 14 days. Group G was normal control, Group H was given 1 % sodium carboxy

methyl cellulose orally, and Group I was given 100 mg/kg body weight echinacea extract (Product-X®), as positive control, orally. On the 15th day (= day 0) and 25th day 0.2 ml L monocytoenes containing 5×10^3 cfu/ml are injected intraperitoneally to all the mice. On day 21 (37th day) after injection the mice were sacrificed and the peritoneal macrophages were harvested for phagocytosis and nitric oxide assays, while the lymphocytes were isolated from the spleen for proliferation assay. All procedures related with animal experimentations were approved by The Central Integrated Research (LPPT) Gadjah Mada University Indonesia number: 068/KEC-LPPT/VII/2012. The equipment, including handling and sacrificing of the animals were in accordance with European Council Legislation 87/609/EEC for the protection of experimental animals [5].

Isolation of compounds

Red betel leaf methanolic extract was fractionated by vacuum liquid chromatography (VLC) method. Isolated compounds (Pc-1 and Pc-2) were purple spots at UV 254 nm, no color at UV 366 nm, and brown colour with cerium sulfate detection. These compounds were eluted using chloroform : ethyl acetate (9:1) mobile phase with 0.7 as the retardation factor (Rf) of Pc-1 and 0.3 as the Rf of Pc-2. Pc-1 and Pc-2 were isolated from the third and fourth fractions of VLC separation using preparative Thin Layer Chromatography (TLC). The spot of the compound was scraped, collected, and then diluted with chloroform : methanol (1:1). The compound was obtained in the form of a crystal after filtration and evaporation.

Macrophage phagocytosis assay

The macrophage phagocytic assay was conducted according to the method of Leijh *et al* method [6] using latex beads with a diameter of 13 μ m. Latex beads were suspended in PBS so that concentration obtained was 2.5×10^7 /ml. Macrophage cultured a day before was washed twice with RPMI 1640 prior to be placed in 24 well plate. The latex beads (200 μ L) were added each well, and then incubated in CO₂ incubator at 37 °C for 60 min. Cells were washed with PBS three times to remove the remaining latex beads. Cover slips containing macrophages were dried at room temperature and fixed with methanol for 30 s. Subsequently, methanol was removed and cover slips containing macrophages were dried and stained with 20 % Giemsa for 30 min. Coverslips were washed with distilled water thoroughly (4-5 times), removed from the culture

wells and dried at room temperature. Activated macrophages were calculated using a light microscope with magnification of 400x. Phagocytic activity was measured by the latex-bead phagocytosis index (PI), the phagocytosis percentage (PP), and the phagocytosis efficiency (PE) [7].

Nitric oxide (NO) assay

A total of 100 μL macrophage cell culture, that have been incubated overnight, were put in 96 well plate. Gries solution (100 μL) was added to each well, incubated for 10 min and then the optical density was read with Elisa reader at 550 nm. Nitric oxide with concentration ranging from 0.078 μM to 20 μM was used as standard [8].

Lymphocytes proliferation assay

Lymphocytes were cultured in 96 well microplate with a volume of 100 μL /well. Ten microlitre of 50 $\mu\text{g}/\text{ml}$ phytohaemagglutinin (PHA) was added to each well, and incubated in a CO₂ incubator at 37 °C, for 72 h. Ten microlitre of 5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), was then added to each well and incubated at 37 °C, 5 % CO₂ for 4 h. The reaction was stopped by adding 100 μL /well 0.04 M HCl-isopropanol. The resulting color was read using an Elisa reader at 550 nm.

Histopathological examination of liver and kidney

Murine peritoneum sheath was opened, after the isolation of peritoneal macrophages and lymphocytes from the spleen. Kidneys and liver were removed and then immersed in 10 % buffered formaline for histopathological examination. Subsequently, the kidney and liver were cut to 4 μm thickness using microtome, and stained using hematoxylin-eosin (HE). Histology slides were examined under a microscope at a magnification of 100x [9].

Statistical analysis

Data analysis was carried out using IBM Statistical Product and Service Solutions (SPSS) statistics 19, and the data were expressed as mean \pm SE. The significance level of treatment effect was determined by one-way analysis of variance (ANOVA) followed by Tukey's test post hoc analysis; p -values less than 0.05 were considered statistically significant.

RESULTS

Isolated compounds

The yield from 8.26 kg of wet red betel leaf was 1.9 kg of dry powder. Using the maceration method, the 1.9 kg dry powder yielded 224.03 g of methanolic extract [10]; 2.12 g of this red betel leaf methanolic extract extracted according to Kustiawan [4] produced 12.0 mg of Pc-1 and 12.1 mg Pc-2 (Figure 1). As can be seen in Figure 1, Pc-2 differs from Pc-1 at their C₇ binding group; Pc-1 binds acetyl while Pc-2 binds hydroxyl group.

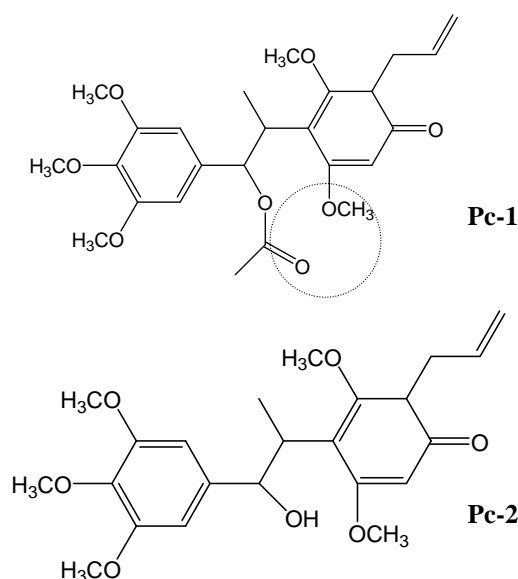


Figure 1: Neolignans isolated from Red betel (*Piper crocatum* Ruiz & Pav); Pc-1 is 2-allyl-4-(1'-acetyl-1'-(3',4',5'-trimethoxyphenyl)propan-2'-yl)-3,5-dimethoxycyclohexa-3,5-dienone; Pc-2 is 2-allyl-4-(1'-hydroxy-1'-(3',4',5'-trimethoxyphenyl)propan-2'-yl)-3,5-dimethoxycyclohexa-3,5-dienone [4].

Immunomodulatory effect

Both compounds isolated from the leaves of red betel (*Piper crocatum* Ruiz & Pav) significantly increased ($p < 0.05$) the phagocytosis percentage and phagocytosis index of peritoneal macrophages of mice infected with *Listeria monocytogenes*. Treatment with Pc-1 and Pc-2 at a dose of 5 or 10 mg/kg body weight showed significant difference in NO production compared to that of normal, solvent and positive control, whereas at a dose of 2.5 mg/kg body weight showed significant differences ($p < 0.05$) compared to the normal control and solvent control groups. However lymphocyte proliferation assay showed no significant difference between Pc-1 and Pc-2, at doses of 2.5, 5, and 10 mg/kg body weight compared to normal control and solvent control groups ($p > 0.05$). Therefore, Pc-

Table 1: Immunomodulatory effect of Pc-1 and Pc-2 at 21st day after the mice were induced by *L. monocytogenes*

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

Values are presented as mean \pm SE ($n = 3$); * $p < 0.05$ was considered to be significant when compared to normal and solvent controls

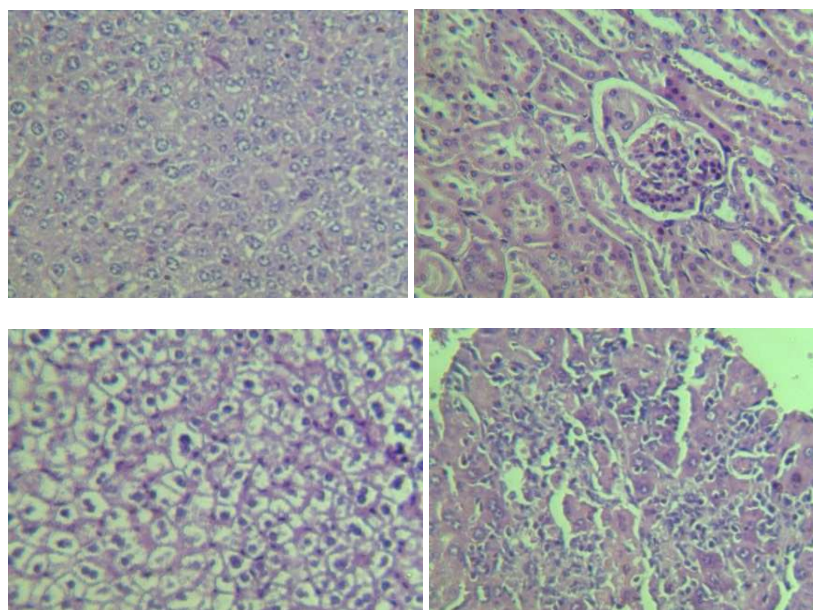


Figure 2: Photomicrography of liver and kidney of Balb/c mice given Pc-1 and Pc-2 at 21st day. A. normal liver, B. normal kidney, C. hydropic degeneration of liver cells, D. necrotic liver cells.

1 and Pc-2 did not have immunomodulatory effect on lymphocyte proliferation (Table 1).

Histopathological effect on liver and kidney

Figure 2 demonstrates the histopathological features of liver and kidney given Pc-1 and Pc-2 isolated from red betel. All groups receiving Pc-1 at doses of 2.5, 5, and 10 mg/kg body weight showed normal histological features of liver and kidney. The group that received Pc-2 showed normal histological feature of kidney. However, mild hydropic degeneration occurred in the group given 2.5 mg/kg body weight Pc-2, whereas, severe hydropic degeneration occurred in the group given 5 mg/kg body weight and 10 mg/kg body weight Pc-2. Necrotic liver cells were also found in the centro lobule given 5 mg/kg body weight (Figure 2).

DISCUSSION

Pc-1 and Pc-2 showed similar profiles as immunomodulators. At a dose of 10 mg/kg body weight both Pc-1 and Pc-2 increased phagocytosis percentage and phagocytosis index. Statistical analysis showed a significant difference ($p < 0.05$), in the macrophage phagocytosis percentage and phagocytosis index in group treated with 10 mg/kg body weight Pc-1 compared with the normal, solvent, and positive (echinacea product) control groups. At a dose of 5 mg/kg body weight, both Pc-1 and Pc-2 are able to increase the phagocytosis percentage and phagocytosis index significantly ($p < 0.05$), both in the normal control and the solvent control group, but not the positive control group. At a dose of 10 mg/kg body weight, macrophage phagocytic capacity of the group given Pc-2 was greater than that of Pc-1 at the same dose.

Macrophage phagocytosis efficiency in the group given Pc-2 at a dose of 5 mg/kg body weight and 10 mg/kg body weight showed a significant difference, whereas Pc-1 was only significantly different at a dose of 10 mg/kg body weight.

In this study, 2.12 g *Piper crocatum* Ruiz & Pav. methanolic extract contained 12.0 mg Pc-1 and 12.1 mg Pc-2. Therefore, the dose of 5 mg/kg body weight Pc-1 is equal to 876 mg/kg body weight extract and the dose of 5 mg/kg body weight Pc-2 is equal to 883 mg/kg body weight extract. Sunila and Kuttan [11] reported that alcoholic extract of *Piper longum* Linn. (10 mg/dose/animal) as well as piperine (1.14 mg/dose/animal) have immunomodulatory activity. Compared with our result in this study, it might be that the neolignans isolated from *Piper crocatum* Ruiz & Pav methanolic extract are less active compared to piperine, and the extract is less active than methanolic extract of *Piper longum* Linn.

The NO level is low (0.08 μ M) in the group treated with both neolignan Pc-1 and Pc-2, even though the phagocytosis percentage and index were relatively high. There was no significant difference between Pc-1 and Pc-2 on the lymphocytes proliferation (Table 1). Kanjwani et al [12] reported the cellular and humoral response activity of *Piper betle* L methanolic extract. Mechanisms of action of several herbal medicines as immunostimulants still unclear [13]. Some medicinal plants may stimulate the immune system whereas some others may suppress the immune response. Various secondary metabolites exhibit a wide range of immunomodulating activity [14]. In this study the two neolignans isolated from *Piper crocatum* Ruiz and Pav increased the phagocytosis percentage and phagocytosis index of macrophage. Macrophage play an important role in innate and adaptive immunity, therefore, the two neolignans might influence the innate and adaptive immunities.

Neolignan structure-activity relationship (SAR) studied by Kong et al [15] stated that at least one free hydroxyl group was essential for the induction of cytotoxicity. Histopathological study of Pc-1 neolignans did not show any abnormal histopathological feature of liver and kidney. Liver damage was observed in the Pc-2 treatment group. Considering this result and the structure similarity of these two neolignans (Pc-1 and Pc-2), an interesting presumption can be brought up that the -OH functional group (Pc-2) might be responsible for the toxicity of the liver damage. In this study, we used a dose of 2.5 mg/kg body weight Pc-2 is equal to 438 mg/kg

body weight extract and resulted in mild hydropic degeneration of the liver. Moreover, Umoh et al [16] found that ethanolic extract of *Piper guineense* at the dose of 20 mg/kg body weight and above is a risk factor for hepatic function impairment and the associated disorder. There is no report about *in vivo* application of *Piper crocatum* Ruiz & Pav. extract, therefore the need for further research dose recommendation.

CONCLUSION

The Pc-1 and Pc-2 isolated from the leaves of *P. crocatum* Ruiz & Pav increased macrophage phagocytosis and nitric oxide production, but not lymphocytes proliferation. There is no abnormal histopathological features found in the kidney due to administration of both compounds. Administration of Pc-2 but not Pc-1 resulted in hydropic degeneration and necrosis to the liver cells.

ACKNOWLEDGEMENT

The first named author is grateful to Dirjend DIKTI Republik Indonesia for financial support for the work by PhD research grant.

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