Title : In-vivo Immunomodulatory Effect and Histopathological of Mice Liver and Kidney Given Two Neolignans Isolated from Red Betel (Piper crocatum Ruiz & Pav.)

Journal name: Tropical Journal of Pharmaceutical Research

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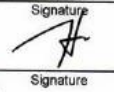
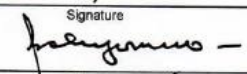
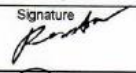
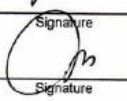
Yustina Sri Hartini		July 6, 2014
Subagus Wahyuono		July 07, 2014
Sitarino Widyarini		July 7, 2014
Agustinus Yuswanto		
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In-vivo Immunomodulatory Effect and Histopathological Features of Mouse Liver and Kidney Treated with Neolignans Isolated from Red Betel (*Piper crocatum* Ruiz & Pav) Leaf

Yustina Sri Hartini[1]*, Subagus Wahyuono[2], Sitarina Widyarini[3] and Agustinus Yuswanto[2]

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ABSTRACT

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

Methods: Balb/c mice immune response were 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 crocatidin and deacetyl crocatidin significantly increased the activity and the capacity of macrophages ($p < 0.05$). Crocatidin and deacetyl crocatidin increase phagocytic activity of macrophage, respectively for 25% and 23% at the dose of 5 mg/kg body weight, and increase the phagocytic index respectively for 38 and 52. Increasing nitric oxide production due to crocatidin and deacetyl crocatidin (2.5, 5, and, 10 mg/kg body weight) was also observed although no lymphocyte proliferation effect was observed. Histopathological examination of liver and kidney of mice given crocatidin demonstrated normal features. On the other hand, hydropic degeneration and liver necrosis were seen in mice given deacetyl crocatidin treatment. Based on this result and the structure similarity of the two compounds (crocatidin and deacetyl crocatidin), an interesting presumption can be made that the –OH functional group (deacetyl crocatidin) was responsible for the toxicity that caused liver damage.

Conclusion: The two neolignans (crocatidin and deacetyl crocatidin) 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. Histophatological features of liver given deacetyl crocatidin demonstrate hydropic degeneration and necrosis, possibly due to the –OH group on deacetyl crocatidin.

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

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].

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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 authenticateded by Wahyono of the Department of Biology, Faculty of Pharmacy, Gadjah Mada University, Yogyakarta, Indonesia

and a voucer specimen (no. BF/284/Ident/Det/VIII/2011) was deposited in herbarium unit at The Faculty of Pharmacy, Sanata Dharma University, Indonesia.

Commented [AO3]: No, it should not deposited in a lab but in a proper herbarium

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 monocytogenes* containing 5 × 10^{3} cfu/ml are injected intraperioneally to all the mice. On day 21 (37th day) after injection the mice were sacrificed and the peritoneal macrophages were harvested for phagocytocis 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].

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Isolation of compounds

Red betel leaf methanolic extract was fractionated by vacuum liquid chromatography (VLC) method. Isolated compounds (crocatidin and deacetyl crocatidin) were purple spots at UV 254 nm, no color at UV 366 nm, and brown colour with cerium sulfate detection. These compounds were eluated using chloroform : ethyl acetate (9:1) mobile phase with 0.7 as the retardation factor (Rf) of crocatidin and 0.3 as the Rf of deacetyl crocatidin . Crocatidin and deacetyl crocatidin 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 mm. 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 µL) were added each well, and then incubated in CO_2 incubator at 37 ºC 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 µg/ml phytohaemaglutinin (PHA) was added to each well, and incubated in a CO_2 incubator at 37 °C, for 72 h. Ten microlitre of 5 mg/ml 3-(4,5-dimethyithiazol-2-yl)-2.5-diphenyltetrazolium bromide (MTT), was then added to each well and incubated at 37 °C, 5 % CO_2 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 % bufferred 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].

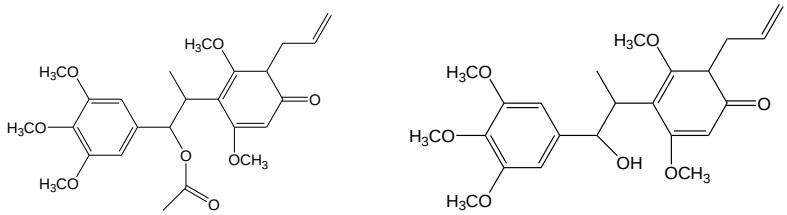
Statistical analysis

Data analysis was carried out using IBM Statistical Product and Service Solutions (SPSS) statistics 19, and the data were expressed as mean ± 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.

RESULT

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 was extracted and produced 224.03 g methanolic extract [10]. Isolation of crocatidin and deacetyl crocatidin from the 2.12 g of red betel leaf methanolic extract produced 12.0 mg of crocatidin and 12.1 mg deacetyl crocatidin. Isolation of crocatidin and deacetyl crocatidin from the methanolic extract of red betel leaves was carried out according to Kustiawan [4]. As can be seen in Figure 1, deacetyl crocatidin differs from crocatidin on their C_7 binding group, crocatidin binds acetyl while deacetyl crocatidin binds hydroxyl group.



crocatidin

deacetyl crocatidin

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

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 crocatidin and deacetyl crocatidin 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.

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

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Group	Phagocytosis (%)	Phagocytosis index	Phagocytosis efficiency	NO production (µM)	Lymphocyte proliferation (OD)
Pc-1 (2.5 mg/kg)	17.2 ± 0.5	26.9 ± 1.1	1.57 ± 0.03	0.077 ± 0.000*	0.069 ± 0.001
Pc-1 (5 mg/kg)	25.1 ± 2.6*	38.2 ± 3.6*	1.53 ± 0.01	0.081 ± 0.000*	0.056 ± 0.006
Pc-1 (10 mg/kg)	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/kg)	18.2 ± 1.6	29.6 ± 3.9	1.65 ± 0.26	0.078 ± 0.000*	0.061 ± 0.001
Pc-2 (5 mg/kg)	22.8 ± 0.7*	52.2 ± 3.2*	2.29 ± 0.07*	0.079 ± 0.000*	0.065 ± 0.003
Pc-2 (10 mg/kg)	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 (n = 3); *p < 0.05 was considered to be significant when compared to normal and solvent controls*

However lymphocyte proliferation assay showed no significant difference between croactidin and deacetyl crocatidin, at doses of 2.5, 5, and 10 mg/kg body weight compared to normal control and solvent control groups ($p > 0.05$). Therefore, croactidin and deacetyl crocatidin 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 croactidin and deacetyl crocatidin isolated from red betel. All groups receiving crocatidin at doses of 2.5, 5, and 10 mg/kg body weight showed normal histological features of liver and kidney. The group that received deacetyl crocatidin showed normal histological feature of kidney. However, mild hydropic degeneration occured in the group given 2.5 mg/kg body weight deacetyl crocatidin, whereas, severe hydropic degeneration occured in the group given 5 mg/kg body weight and 10 mg/kg body weight deacetyl crocatidin. Necrotic liver cells were also found in the centro lobule given 5 mg/kg body weight (Figure 2).

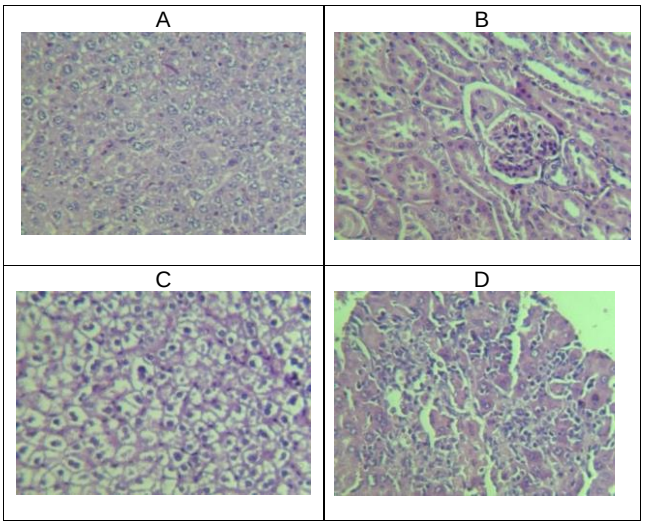

Figure 2: Photomicrography of liver and kidney of Balb/c mice given croactidin and deacetyl crocatidin at 21st day. A. normal liver, B. normal kidney, C. hydropic degeneration of liver cells, D. necrotic liver cells.

DISCUSSION

Croactidin and deacetyl crocatidin showed similar profiles as immunomodulators. At a dose of 10 mg/kg body weight both croactidin and deacetyl crocatidin 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 croactidin compared with the normal, solvent, and positive (echinacea product) control groups. At a dose of 5 ml/kg body weight, both croactidin and deacetyl crocatidin 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 deacetyl crocatidin was greater than that of crocatidin at the same dose. Macrophage phagocytosis efficiency in the group given deacetyl crocatidin at a dose of 5 mg/kg body weight and 10 mg/kg body weight showed a significant difference, whereas crocatidin 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 crocatidin and 12.1 mg deacetyl crocatidin. Therefore, the dose of 5 mg/kg body weight deacetyl crocatidin is equal to 876 mg/kg body weight extract and the dose of 5 mg/kg body weight crocatdin is equal to 883 mg/kg bodyweight 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.

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The NO level is low (0.08 µM) in the group treated with both neolignan croactidin and deacetyl crocatidin, even though the phagocytosis percentage and index were relatively high. There were no significant difference of both croactidin and deacetyl crocatidinon the lymphocytes proliferation (Table 1). Kanjwani *et al* [12] reported the cellular and humoral response activit 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 & 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.

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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 crocatidin neolignans did not show any abnormal histopathological feature of liver and kidney. Liver damage was observed in the deacetyl crocatidin treatment group. Considering this result and the structure similarity of these two neolignans (croactidin and deacetyl crocatidin), an interesting presumption can be brought up that the –OH functional group (deacetyl crocatidin) might be responsible for the toxicity of the liver damage. In this study, we used a dose of 2.5 mg/kg body weight deacetyl crocatidin 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 croactidin and deacetyl crocatidin 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 deacetyl crocatidin but not crocatidin resulted in hydropic degeneration and necrotis 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.

Commented [AO8]: What is this and what does it mean? Can't you use the English translation?

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

Yustina Sri Hartini[1]*, Subagus Wahyuono[2], Sitarina Widyarini[3] and Agustinus Yuswanto[2]

[1]Faculty of Pharmacy, Sanata Dharma University, Paingan Maguwoharjo Depok Sleman Yogyakarta [2]Faculty of Pharmacy, Gadjah Mada University, [3]Faculty of Veterinary Medicine, Gadjah Mada University, Bulaksumur, Yogyakarta, Indonesia.

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ABSTRACT

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

Methods: Balb/c mice immune response were 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 crocatidin and deacetyl crocatidin significantly increased the activity and the capacity of macrophages ($p < 0.05$). Crocatidin and deacetyl crocatidin increase phagocytic activity of macrophage, respectively for 25% and 23% at the dose of 5 mg/kg body weight, and increase the phagocytic index respectively for 38 and 52. Increasing nitric oxide production due to crocatidin and deacetyl crocatidin (2.5, 5, and, 10 mg/kg body weight) was also observed although no lymphocyte proliferation effect was observed. Histopathological examination of liver and kidney of mice given crocatidin demonstrated normal features. On the other hand, hydropic degeneration and liver necrosis were seen in mice given deacetyl crocatidin treatment. Based on this result and the structure similarity of the two compounds (crocatidin and deacetyl crocatidin), an interesting presumption can be made that the –OH functional group (deacetyl crocatidin) was responsible for the toxicity that caused liver damage.

Conclusion: The two neolignans (crocatidin and deacetyl crocatidin) 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. Histophatological features of liver given deacetyl crocatidin demonstrate hydropic degeneration and necrosis, possibly due to the –OH group on deacetyl crocatidin.

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

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.

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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 authenticateded by Wahyono of the Department of Biology, Faculty of Pharmacy, Gadjah Mada University, Yogyakarta, Indonesia

and a voucer specimen (no. BF/284/Ident/Det/VIII/2011) was deposited in herbarium unit at The Faculty of Pharmacy, Sanata Dharma University, Indonesia.

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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 monocytogenes* containing 5 × 10^{3} cfu/ml are injected intraperioneally to all the mice. On day 21 (37th day) after injection the mice were sacrificed and the peritoneal macrophages were harvested for phagocytocis 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].

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Isolation of compounds

Red betel leaf methanolic extract was fractionated by vacuum liquid chromatography (VLC) method. Isolated compounds (crocatidin and deacetyl crocatidin) were purple spots at UV 254 nm, no color at UV 366 nm, and brown colour with cerium sulfate detection. These compounds were eluated using chloroform : ethyl acetate (9:1) mobile phase with 0.7 as the retardation factor (Rf) of crocatidin and 0.3 as the Rf of deacetyl crocatidin . Crocatidin and deacetyl crocatidin 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 mm. 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 µL) were added each well, and then incubated in CO_2 incubator at 37 ºC 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 µg/ml phytohaemaglutinin (PHA) was added to each well, and incubated in a CO_2 incubator at 37 °C, for 72 h. Ten microlitre of 5 mg/ml 3-(4,5-dimethyithiazol-2-yl)-2.5-diphenyltetrazolium bromide (MTT), was then added to each well and incubated at 37 °C, 5 % CO_2 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 % bufferred 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].

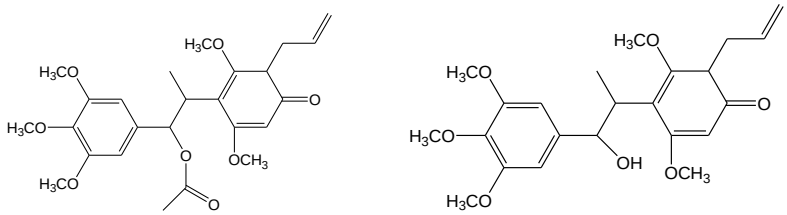
Statistical analysis

Data analysis was carried out using IBM Statistical Product and Service Solutions (SPSS) statistics 19, and the data were expressed as mean ± 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.

RESULT

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 was extracted and produced 224.03 g methanolic extract [10]. Isolation of crocatidin and deacetyl crocatidin from the 2.12 g of red betel leaf methanolic extract produced 12.0 mg of crocatidin and 12.1 mg deacetyl crocatidin. Isolation of crocatidin and deacetyl crocatidin from the methanolic extract of red betel leaves was carried out according to Kustiawan [4]. As can be seen in Figure 1, deacetyl crocatidin differs from crocatidin on their C_7 binding group, crocatidin binds acetyl while deacetyl crocatidin binds hydroxyl group.



crocatidin

deacetyl crocatidin

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

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 crocatidin and deacetyl crocatidin 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.

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

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Group	Phagocytosis (%)	Phagocytosis index	Phagocytosis efficiency	NO production (µM)	Lymphocyte proliferation (OD)
Pc-1 (2.5 mg/kg)	17.2 ± 0.5	26.9 ± 1.1	1.57 ± 0.03	0.077 ± 0.000*	0.069 ± 0.001
Pc-1 (5 mg/kg)	25.1 ± 2.6*	38.2 ± 3.6*	1.53 ± 0.01	0.081 ± 0.000*	0.056 ± 0.006
Pc-1 (10 mg/kg)	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/kg)	18.2 ± 1.6	29.6 ± 3.9	1.65 ± 0.26	0.078 ± 0.000*	0.061 ± 0.001
Pc-2 (5 mg/kg)	22.8 ± 0.7*	52.2 ± 3.2*	2.29 ± 0.07*	0.079 ± 0.000*	0.065 ± 0.003
Pc-2 (10 mg/kg)	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 (n = 3); *p < 0.05 was considered to be significant when compared to normal and solvent controls*

However lymphocyte proliferation assay showed no significant difference between croactidin and deacetyl crocatidin, at doses of 2.5, 5, and 10 mg/kg body weight compared to normal control and solvent control groups ($p > 0.05$). Therefore, croactidin and deacetyl crocatidin 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 croactidin and deacetyl crocatidin isolated from red betel. All groups receiving crocatidin at doses of 2.5, 5, and 10 mg/kg body weight showed normal histological features of liver and kidney. The group that received deacetyl crocatidin showed normal histological feature of kidney. However, mild hydropic degeneration occured in the group given 2.5 mg/kg body weight deacetyl crocatidin, whereas, severe hydropic degeneration occured in the group given 5 mg/kg body weight and 10 mg/kg body weight deacetyl crocatidin. Necrotic liver cells were also found in the centro lobule given 5 mg/kg body weight (Figure 2).

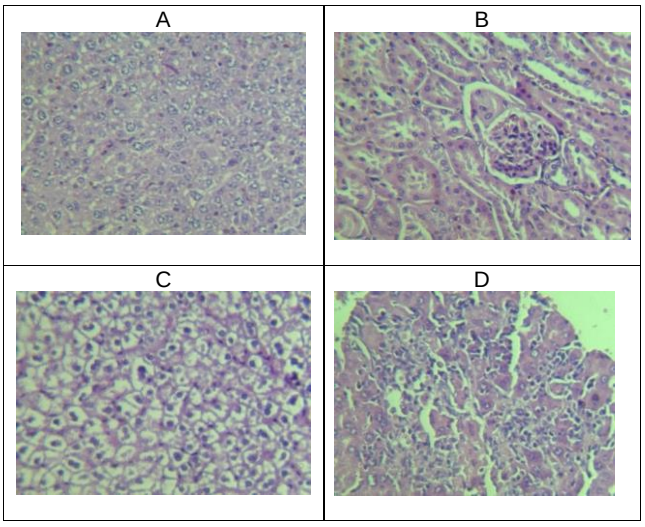

Figure 2: Photomicrography of liver and kidney of Balb/c mice given croactidin and deacetyl crocatidin at 21st day. A. normal liver, B. normal kidney, C. hydropic degeneration of liver cells, D. necrotic liver cells.

DISCUSSION

Croactidin and deacetyl crocatidin showed similar profiles as immunomodulators. At a dose of 10 mg/kg body weight both croactidin and deacetyl crocatidin 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 croactidin compared with the normal, solvent, and positive (echinacea product) control groups. At a dose of 5 ml/kg body weight, both croactidin and deacetyl crocatidin 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 deacetyl crocatidin was greater than that of crocatidin at the same dose. Macrophage phagocytosis efficiency in the group given deacetyl crocatidin at a dose of 5 mg/kg body weight and 10 mg/kg body weight showed a significant difference, whereas crocatidin 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 crocatidin and 12.1 mg deacetyl crocatidin. Therefore, the dose of 5 mg/kg body weight deacetyl crocatidin is equal to 876 mg/kg body weight extract and the dose of 5 mg/kg body weight crocatdin is equal to 883 mg/kg bodyweight 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.

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The NO level is low (0.08 µM) in the group treated with both neolignan croactidin and deacetyl crocatidin, even though the phagocytosis percentage and index were relatively high. There were no significant difference of both croactidin and deacetyl crocatidinon the lymphocytes proliferation (Table 1). Kanjwani *et al* [12] reported the cellular and humoral response activit 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 & 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.

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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 crocatidin neolignans did not show any abnormal histopathological feature of liver and kidney. Liver damage was observed in the deacetyl crocatidin treatment group. Considering this result and the structure similarity of these two neolignans (croactidin and deacetyl crocatidin), an interesting presumption can be brought up that the –OH functional group (deacetyl crocatidin) might be responsible for the toxicity of the liver damage. In this study, we used a dose of 2.5 mg/kg body weight deacetyl crocatidin 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 croactidin and deacetyl crocatidin 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 deacetyl crocatidin but not crocatidin resulted in hydropic degeneration and necrotis 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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July 2, 2014

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Faculty of Pharmacy
Sanata Dharma University
Paingan Maguwoharjo Depok Sleman Yogyakarta, Indonesia

Dear Dr Hartini:

RE: MANUSCRIPT TITLED *"IN-VIVO IMMUNOMODULATORY EFFECT AND HISTOPATHOLOGICAL FEATURES OF MICE LIVER AND KIDNEY GIVEN TWO NEOLIGNANS ISOLATED FROM RED BETEL (PIPER CROCATUM RUIZ & PAV.)"*

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