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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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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 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 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.*

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 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.

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 15^{th} day (= day 0) and 25^{th} day 0.2 ml L monocytogenes containing 5×10^3 cfu/ml are injected intraperioneally to all the mice. On day 21 (37^{th} 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].

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 CO2 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.

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 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 C7 binding group, crocatidin binds acetyl while deacetyl crocatidin binds hydroxyl group.

DISCUSSION

Morinda citrifolia (MC) is one of the traditional medicines used to treat cardiovascular diseases such as hyperlipidemia. As MC is reported to be rich in flavonoids, a polyphenol substance, which could inhibit lipid biosynthesis [19] especially by inhibiting the HMG Co-A reductase [20]. Various studies, both in humans and animals, have shown similar results in reducing effect to TC and LDL-C. Mandukhail [14] conducted a research in rats that injected high dose of lipids and the results showed that MC extract inhibited biosynthesis, absorption and secretion of lipids on day 10. Besides that, Subramanian and Rao [21] gave a result that MC might act as an anti-diabetic, anti-hyperlipidemia and anti-oxidant in diabetic rats. In humans, Wang *et al* [15] found that 68 smoking volunteers who were given MC two times a day for 30 days showed reduced levels of TC and LDL-C. All the studies mentioned above produced similar results with this study. In this study, MCC reduced serum TC and LDL-C level because there were significant decreases in TC and LDL-C level ($p < 0.05$).

From this study, sex did not give a significant decrease in TC level. Theoretically, before the age of 50, TC in males might be higher than in females at the same age. But, after the age of 50 the reverse is the case. It is caused by hormonal effect in females after menopause. After menopause (natural or surgical) TC would rise so LDL-C rises too [5]. However, there were some studies which showed that sex did not affect the TC reduction. John *et al* [25] in his research stated that control of dyslipidemia did not vary significantly across ethnic-sex group and

prevalence of dyslipidemia did not differ significantly between women and men. Cooke and Hammerash [26] also reported the same. They showed that there were no significant sex differences in potency groups to manage dyslipidemia. Another study by Syed *et al* [27] showed that there were no significant reductions in serum TC and LDC-C levels in both male and female patients ($p > 0.05$).

Another factor was BMI. In this study BMI gave OR = 6.2 and $p = 0.0001$. This result shows that there was a significant correlation between BMI and TC reduction. Freeman and Junge [5] stated that high BMI or body weight increases the risk of a higher TC level. Denke [28] explained that there was a relationship between body weight changes and serum TC level among people between adults and middle age.

Exercising regularly could lower the levels of TC, LDL-C, and triglycerides in humans [29]. This study shows a significant difference between patients who exercised regularly and those who did not (OR = 14; $p = 0.001$). Kuriyan *et al* [30] produced same results with this study. They explained that physical activity reduces most of the artherosclerotic risk factors and regular exercise has been shown to reduce LDL-C and triglycerides.

Lifestyle modification to normalize body weight and having healthy patterns of dietary intake might give a significant result in serum TC reduction. Individualized dietary intake for reducing TC level in blood have shown to be modestly effective. Diets low in saturated fat and cholesterol could lower LDL-C [30]. Our study showed that diet could decrease TC level. A comparison between patients with a good diet and those without showed significant differences in TC reduction (OR = 16.3; $p = 0.0001$).

One other factor that influenced the reduction of TC level was smoking habits (OR = 14; $p = 0.001$). A cigarette contains many toxins such as tar, nicotine and carbon monoxide. Smoking can decrease the oxygen serum level and lead to elevation in heart rate, reduction in high density lipids (HDL) and damage of endothelium [31]. Jacobson [32] stated that there was a correlation between smoking and TC level while Schultemaker [33] explained that there was a significant difference in the TC levels of patients who smoked and those who do not. Shi-Dou Lin *et al* [34] also found that TC level is related to smoking habits. Thus, the findings are buttressed those of the cited above, especially with regard to correlation between smoking habit and reduction in TC level.

No serious adverse effects were reported during the present study. Adverse events were just limited to mild gastrointestinal symptoms such as flatulence and abdominal distention after the first few doses but the symptoms subsided within a week in all subjects.

CONCLUSION

The results indicate that 1 g extract, when given orally three times a day, significantly reduces TC and LDL-C levels. It seems that consumption of MCC when combined with suitable control of some factors such as age, BMI, diet, exercise and smoking habit significantly reduces TC levels in patients. The results from this study justify the medicinal use of *Morinda citrifolia* in hypercholesterolemia and may also be of relevance in the treatment of cardiovascular diseases.

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