Title: The comparison of two neolignans isolated from red betel leaf and its extract against macrophage

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> The Comparison of Two Neolignans Isolated from Red Betel Leaf and Its Extract against Macrophage Phagocytic Activity, the Level of AST, and Histopathological Features of the Liver in Mice
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The manuscript "The Comparison of Two Neolignans Isolated from Red Betel Leaf and Its Extract against Macrophage Phagocytic Activity, the Level of AST, and Histopathological Features of the Liver in Mice" has been reviewed. I have carefully gone through the manuscript entitled " The Comparison of Two Neolignans Isolated from Red Betel Leaf and Its Extract against Macrophage Phagocytic Activity, the Level of AST, and Histopathological Features of the Liver in Mice" submitted for the favour of publication in OPEM. Technically this manuscript is meaningful , but I have some concern about this manuscript, I am giving here specific examples for the author's convenience.

1. The abstract section looks like very complicated and no precise sentences. The author should rewrite again the abstract section, including emphasize only results, methods and final conclusion, not descriptive.
2. The author should provide in details methods of the extraction, fractionation, and isolation of Pc-1 and Pc-2. Although the provided some information, this is not enough for how they isolated Pc-1 and Pc-2.
3. The author should include 1H and 13C NMR data of isolated two compounds Pc-1 and Pc-2 in materials and methods section.
4. Although the author explained that Pc-1 and Pc-2 are major compounds in Red Betel Leaf extract, but I strongly recommend the author should provide HPLC chromatogram, that Red Betel Leaf extract contains Pc-1 and Pc-2.
5. The author should rewrite again conclusion section, it should be attractive, that the readers easily understand this article.

Reviewer #2: 1. Have you tested any compounds other than Pc1 and Pc2?
2.Why did you choose the 14-day treatment period?

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There is additional documentation related to this decision letter. To access the file(s), please click the link below. You may also login to the system and click the 'View Attachments' link in the Action column.

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The Comparison of Two Neolignans Isolated from Red Betel Leaf and Its Extract against Macrophage Phagocytic Activity, the Level of AST, and Histopathological Features of the Liver in Mice

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Abstract

A single active compound isolated from a plant extract may show a lower, equal, or greater activity compared to its extract. The two neolignans (Pc-1 and Pc-2) has been isolated from red betel (*Piper crocatum* Ruiz & Pav) leaf. The aim of this study was to compare the phagocytic activity of macrophage and histopathological feature in mice treated with Pc-1, Pc-2 and its extract. *Listeria monocytogenes* was used to induce Balb/c mice immune response. Peroral administration of Pc-1 and Pc-2 and red betel leaf methanolic extract were carried out. The phagocytic effect was determined by macrophage phagocytosis and nitric oxide (NO) assays. The morphological feature of liver and kidney were observed using light microscope. The level of Apartate Transaminase (AST Serum) and Alanine transaminase (ALT serum*)* were also measured from the blood serum before and after *L. monocytogenes* infection. The results shows that the macrophage phagocytic activity of given 450 mg/kg body weight extract was equal to 5 mg/kg body weight of Pc-1 and Pc-2. The activity pattern of Percentage Phagocytosis (PP), Index Phagocytosis (IP), and Efficiency Phagocytosis (EP) of extract, Pc-1, and Pc-2 were similar, as well as the NO production. A certain dose of extract and both isolated compound reduced the level of AST while there were no effect on ALT level. There were no histopathological features differences in the liver after treated with extract and Pc-1. However, Pc-2 treatment caused hydropic degeneration on liver. Therefore it can be concluded that there were equal activity between extract and the isolated compound in certain dose. The extract and isolated compound reduced the level of AST while there were no effect on ALT. The only Pc-2 affected the histopathological features of the liver.

Keywords: Red betel (*Piper crocatum* Ruiz & Pav.), isolated compound, extract, phagocytic, histopathological

Introduction

Genus *Piper* have been used for traditional medicine. One of them is red betel (*Piper crocatum* Ruiz & Pav). The neolignans (Pc-1 and Pc-2) accumulated in the leaves, stems, and flowers of red betel with the highest concentrations in the leaves (Hartini and Nugroho, 2017).The methanol extract of red betel leaf and fractions of the extract is able to increase the activity of macrophage phagocytosis (Hartini et al., 2013). In vitro method showed Pc-1 and Pc-2 from red betel has an effect on macrophage phagocytic activity (Kustiawan, 2012). Our previous study showed that Pc-1 and Pc-2 isolated from the red betel leaf increased macrophage phagocytic activity and nitric oxide (NO) production (Hartini et al., 2014). A plant extract produced a higher or smaller response than those of an equivalent dose of the isolated compound considered to be the 'active' one (Mukherjee, et al., 2009). Therefore, the current research aim to compare the effect of Pc-1, Pc-2 and Pc-extract on macrophage phagocytic activity and histopathological features of the liver in mice.

Material and Methods

Plant material

The red betel (*Piper crocatum* Ruiz & Pav.) fresh leaves were harvested from Tawangmangu, Central of Java, Indonesia. Plant species identification was done by Wahyono, Department of Biology, Faculty of Pharmacy, Universitas Gadjah Mada, Yogyakarta, Indonesia. The voucer speciemn was stored in herbarium unit at The Faculty of Pharmacy, Sanata Dharma University, Indonesia (no. BF/184/Ident/Det/VI/2011).

Extract preparation

The powdered dried leaves were put in macerator, methanol was added to the macerator until all of the powder was submerged. The mixture was then stired overnight. Liquid immersion results was separated, while the waste was remacerated two times in the same way. Liquid immersion result of three times maceration were collected, then evaporated using a rotary evaporator to obtain a thick extract (Hartini et al., 2013).

Fractionation preparation

Sebanyak 10 gram silika gel 60 ditambahkan sedikit demi sedikit sambil diaduk dalam cawan porselen berisi 2 gram ekstrak yang ditetesi eter sedikit demi sedikit sehingga diperoleh campuran yang homogen dan kering (*free flowing*). Pembuatan kolom dilakukan dengan memasukkan sebanyak 15 gram silika gel sedikit demi sedikit ke dalam *sintered glass Buchner* sambil divakum untuk memperoleh massa fase diam yang kompak dan padat. Serbuk ekstrak *free flowing* dipindahkan sedikit demi sedikit ke dalam *sintered glass Buchner* di atas fase diam dengan permukaan atas diusahakan rata sambil divakum. Bagian atas ditutup dengan 2 lembar kertas saring sebesar diameter kolom. Fraksinasi dilakukan dengan menuang pelarut secara perlahan-lahan pada permukaan kertas saring sambil divakum. Pelarut yang digunakan berturut-turut 50 ml n-heksana, 50 ml kloroform, 50 ml kloroform, 50 ml etil asetat, dan 100 ml metanol untuk mendapatkan fraksi I, II, III, IV, dan V. Hasil fraksinasi ditampung dalam cawan porselen, setelah kering fraksi-fraksi yang didapat ditimbang. Kandungan senyawa didalam masing-masing fraksi diperiksa dengan metode KLT dengan detector spektrofotometer UV Vis pada panjang gelombang 254 dan 366 nm kemudian pemanasan dan penyemprotan dengan reagen serium (IV) sulfat.

Isolation of compounds

The purposes of the compound isolation was to isolate the Pc-1 and Pc-2 in the Pc-extract isolated before. The compound characters 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 Pc-1 and 0.3 as the Rf of Pc-2 . The Pc-extract was fractionated by vacuum liquid chromatography (VLC) method. Pc-1 and Pc-2 were detected in the third and fourth fractions of VLC. Both fractions were then separated using preparative Thin Layer Chromatography (TLC). The detected 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. Melting points of both compounds was determined using Mettler Toledo MP70 and the molecular weight stated from Gas Chromatography-Mass Spectrometry (GC-MS) analysis using caplary column Agilent 19091S-433.

Animals

Eight weeks old male Balb/c mice with 25-30 g bodyweight were used in this research. Mice were grouped into seven groups. Each group consisted of five mice. Groups A and B were treated with the neolignans Pc-1 and Pc-2 at the dose of 5 mg/kg body weight. Groups C, D, and E were treated Pc-extract with the dose of 150, 300, and 450 mg/kg body weight, respectively. Both neolignans and Pc-extract were given orally once in a day for 14 days. Group F was a normal control and Group H was given orally 1 % sodium carboxy methyl cellulose. At 15^{th} day (day 0) and 25^{th} day 0.2 ml *L. monocytogenes* (5×10^3 cfu/ml) injected intraperioneally to all of the mice. Measurement of macrophage phagocytic activity were done before *L. monocytogenes* injection (day 0) and day 37^{th} (day 21) after *L. monocytogenes* injection. All procedures associated with animal experimentations were approved by The Central Integrated Research (LPPT) Universitas Gadjah Mada Indonesia number: 221/KEC-LPPT/III/2015. European Council Legislation 87/609/EEC protocol for the protection of experimental animals was conducted for handling and sacrificing of the animals (Mitjans et al., 2001).

Macrophage phagocytosis assay

The method of Leijh *et al* (1986) using latex beads with a diameter of 13 mm were used for the assay of macrophage phagocytic. Latex beads were suspended in PBS up to the concentration 2.5×10^7/ml. Macrophage cultured a day before was washed twice with RPMI 1640 and to be placed in 24 well plate. The latex beads (200 µL) were added to 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 seconds. The following process was removing the methanol. The cover slips containing macrophages were dried and stained with 20 % Giemsa for 30 min. Coverslips were washed with distilled water thoroughly (4-5 times), separated from the culture wells and dried at room temperature. The calculation of activated macrophages were done using a light microscope with magnification of 400x. The activity of macrophages was measured by the latex-bead phagocytosis index (PI), the phagocytosis percentage (PP), and the phagocytosis efficiency (PE) (Sanchez et al., 2008).

Nitric oxide (NO) assay

The overnight incubated macrophage cell culture with the amount of 100 µL were placed in 96 well plate. With the addition of Gries solution (100 µL) to each well and the incubation for 10 min, the optical density was read using Elisa reader at 550 nm. Concentration ranging from 0.078 µM to 20 µM of NO was used as standard.

Histopathological examination of liver

Peritoneal macrophages were isolated from the spleen, murine peritoneum sheath was opened, and liver were removed. The liver were then immersed in 10 % bufferred formaline for histopathological examination. The kidney and liver were cut to 4 µm thickness using microtome, and stained using hematoxylin-eosin (HE). Histological slides were observed under a light microscope at a magnification of 400x.

AST/ALT assay

The International Federation of Clinical Chemistry (IFCC) method without pyridoxal phosphate (P-5'-P) were done for analysis of Apartate Transaminase (AST)/ Glutamic Oxaloacetic Transaminase (SGOT) and Alanine transaminase (ALT)/ Glutamic Pyruvic Transaminase (SGPT) in serum. The absorbance was measured at 340 nm.

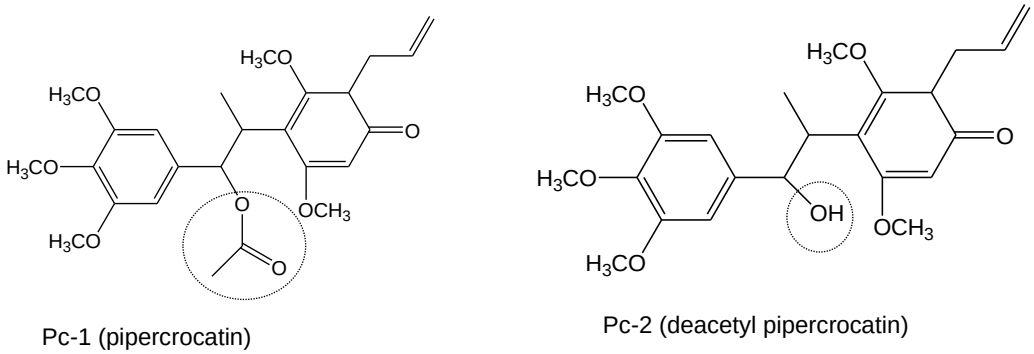
Data analysis

Histopathological features were analysed descriptively, while macrophage phagocytic activity, NO and level of AST/ALT were statistically analysed 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

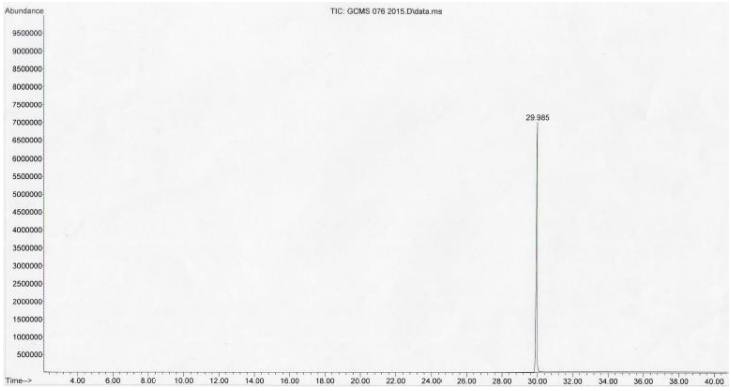
Isolation of Pc-1 and Pc-2 from Pc-extract was carried out according to Hartini et al. (2014). The Pc-1 was elucidated as 2-allyl-4-(1'-acetyl-1'-(3",4",5"-trimethoxyphenyl)propan-2'-yl)-3,5 dimethoxycyclohexa -3,5-dienone; Pc-2 was elucidated as 2-allyl-4-(1'-hydroxy-1'-(3",4",5"-trimethoxyphenyl) propan-2'-yl) -3,5-dimethoxycyclohexa-3, 5-dienone (Kustiawan, 2012). Pc-2 differs from Pc-1 on their C_7 binding group, Pc-1 binds acetyl while Pc-2 binds hydroxyl group (detail structure can be seen in Figure 1). The trivial name for Pc-1 and Pc-2 are pipercrocatin and deacetyl pipercrocatin, respectively.



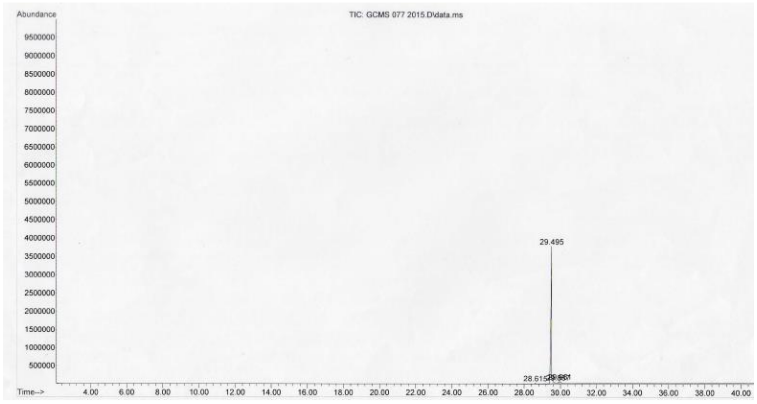
Pc-1 (pipercrocatin) Pc-2 (deacetyl pipercrocatin)

Figure 1. Isolated compounds from red betel (*Piper crocatum*, Ruiz & pav.)

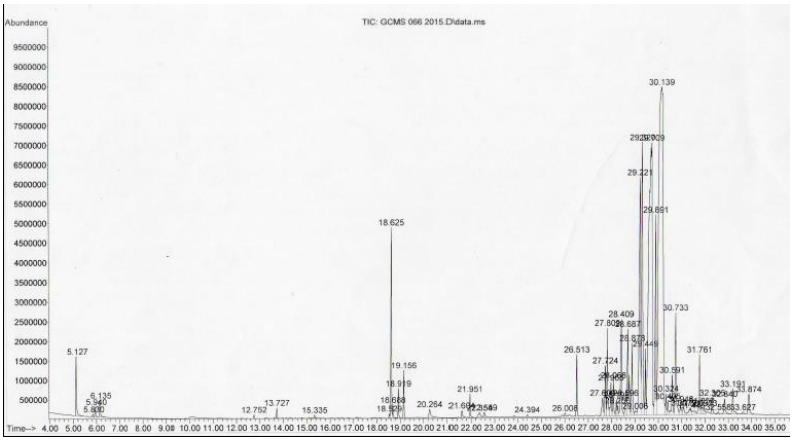
Both compounds in Figure 1 are neolignan which have similar structure each other. The melting point of Pc-1 and Pc-2 were 165-167ºC and 161.5 ºC respectively. The GC-MS spectrogram showed that Pc-1 have molecular weight 460 with retention time at 29.985 minutes (100% of total peak); while Pc-2 have molecular weight 418 with retention time at 29.495 minutes (96.7% of total peak).



Kromatogram GC-MS isolat Pc-1



Kromatogram GC-MS isolat Pc-2



Kromatogram GC-MS Ekstrak daun *Piper crocatum* Ruiz & Pav.

Macrophage Phagocytic Activity

After 14th days treatment (day 0) there was no significant differences in macrophage phagocytic activity (PP, PI, and PE) and NO production among the groups of treatment. The same result were also found when it was compared to negative control. The following experiment, the treated mice were infected with *L. monocytogenes.* At 21th day of infection, the PP and PI showed significantly increased between those of treated group and negative control (Figure 2 and 3).

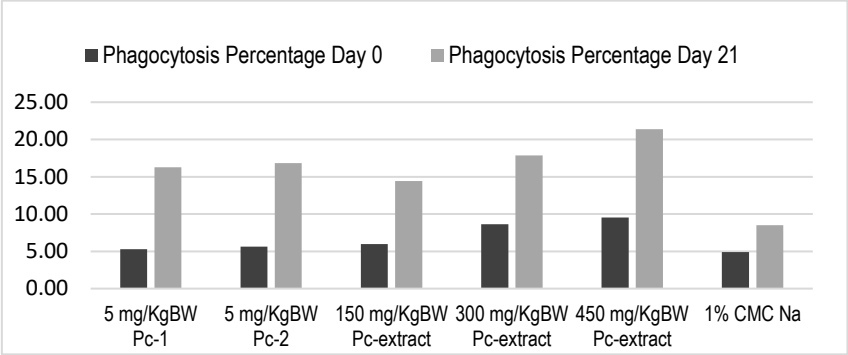


Figure 2. Macrophage Phagocytosis Percentage of mice on day 0 and 21th day after *L. monocytogenes* infection

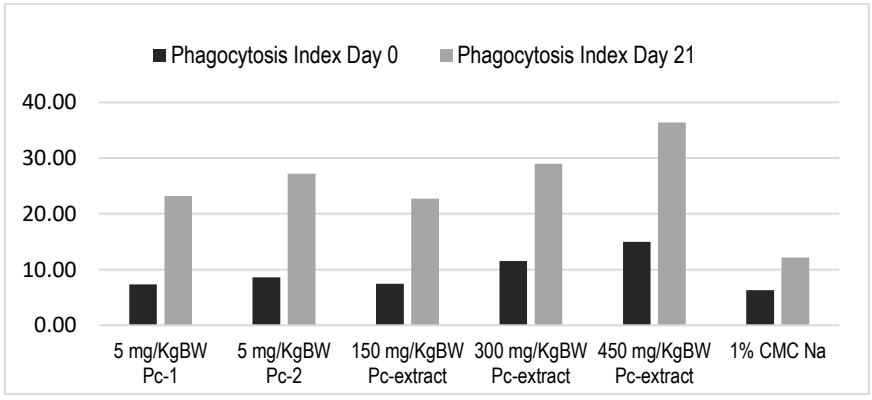


Figure 3. Macrophage Phagocytosis Index of mice on day 0 and 21th day after *L. monocytogenes* infection

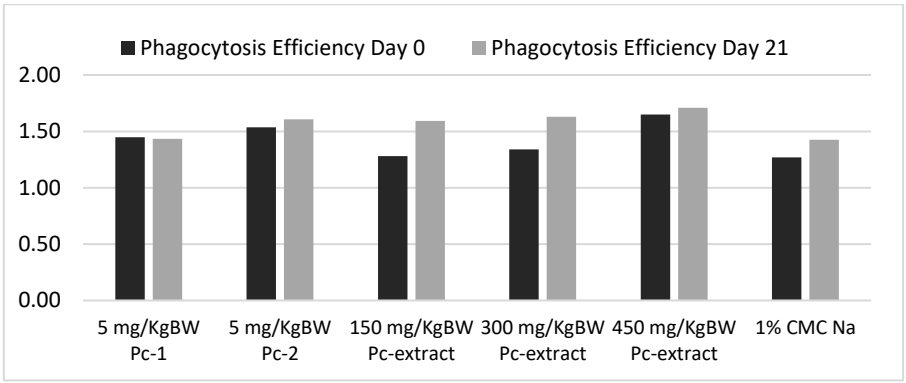


Figure 4. Macrophage Phagocytosis Efficiency of mice on day 0 and 21th day after *L. monocytogenes* infection

The PP and PI of groups treated with the isolates (5 mg/kg body weight Pc-1 and Pc-2) were equal to those of 450 mg/kg body weight Pc-extract. The phagocytic activity on 450 mg/kg body weight Pc-extract seemed more efficient than the neolignans. However statistical analysis showed there is no significantly differences in Pc-extract compare to its PP and PI (Figure 4).

Nitrict Oxide (NO) Production Activity

The results of NO assay showed the similar pattern with macrophage phagocytic activity assay (figure 5). At the dose of 150 mg/kg body weight Pc-extract, the NO production was increased significantly. At the dose of 300 and 450 mg/kg body weight Pc-extract did not increase NO production significantly.

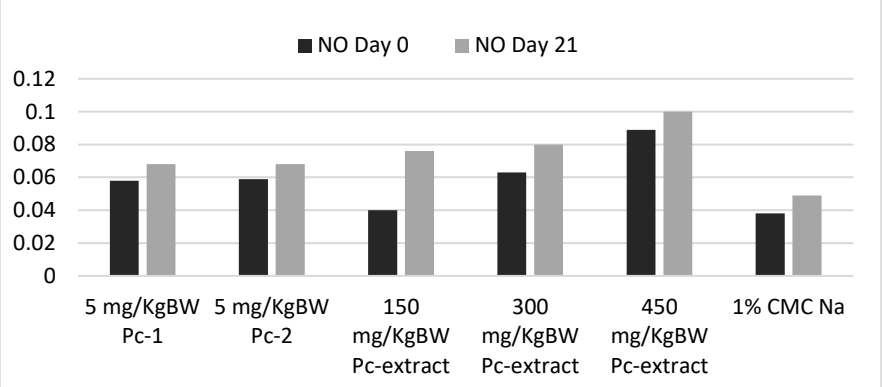


Figure 5. Nitric oxide production of mice on day 0 and 21th day after *L. monocytogenes* infection

The Effect of Neolignans and Pc-extract on The Liver

Table 1. The level of AST and ALT assay of mice blood serum on 21th day after *L. monocytogenes* infection

Group	Treatment	Dose (mg/kg body weight)	AST	ALT
1	Pc-1	5	85 ± 7*	53 ± 5
2	Pc-2	5	93 ± 4*	56 ± 6
3	Pc-extract	150	84 ± 3*	42 ± 5
4	Pc-extract	300	118 ± 11	68 ± 12
5	Pc-extract	450	126 ± 6	53 ± 7
6	Negative/Vehicle control	-	142 ± 4	45 ± 2

Values are presented as mean ± SE (n : 3); *p<0.05 was considered to be significant when compared to negative control

The result of histopathological analysis showed that treatment with Pc-extract dose of 150, 300, and 450 mg/kg body weight did not cause the change of liver histopathological features However Pc-2 resulted in hydropic degeneration of the liver.

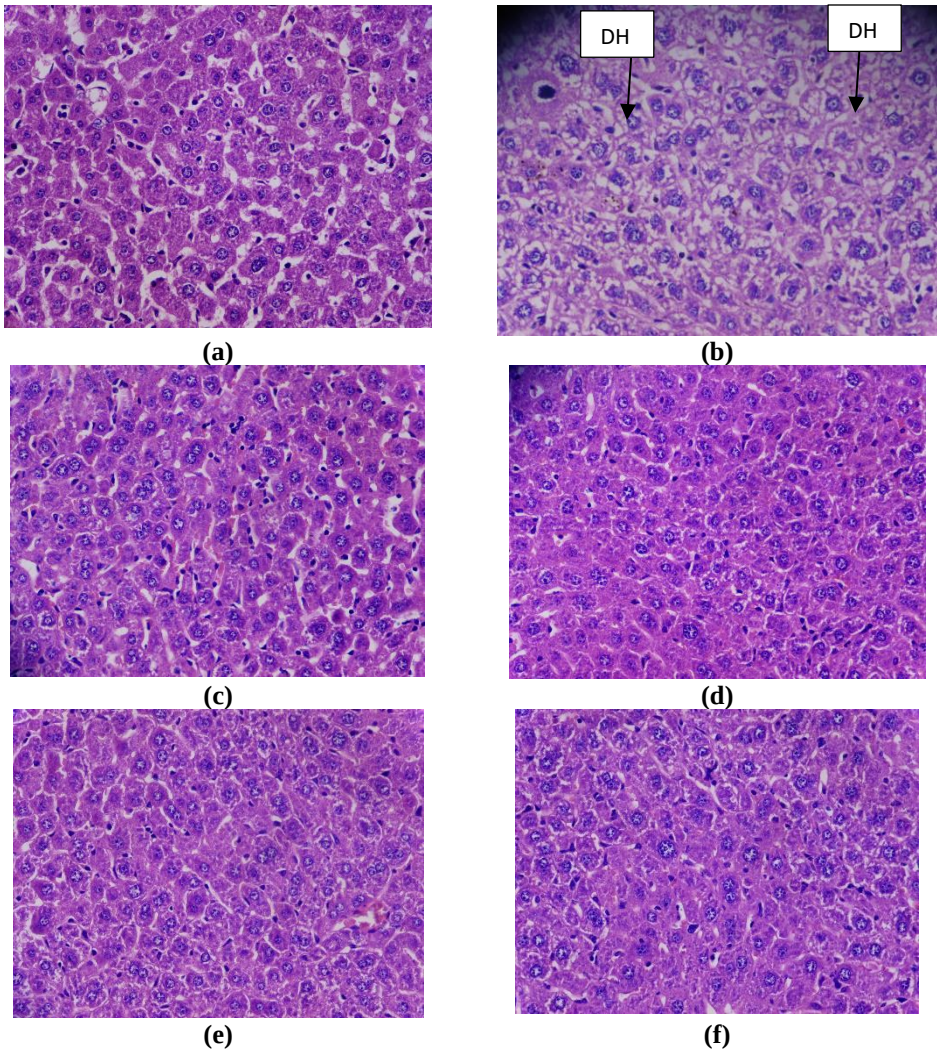


Figure 6. Photomicrography of liver mice treated with *(a)* 5 mg/kg body weight Pc-1; (b) 5 mg/kg body weight Pc-2; (c) 150 mg/kg body weight Pc-extract; (d) 300 mg/kg body weight Pc-extract; (e) 450 mg/kg body weight Pc-extract; (f) CMC Na 1%.

Discussion

Melting point (mp) of Pc-1 and Pc-2 was different from the melting point of neolignan from other Piper species. Neolignan mp from *P. argyrophylum* namely futoquinol and kadsurin B were 97-98^{o} and 101-102^{o} respectively. Compounds with a mp similar to the Pc-1 was an alkaloid isolated from *P. argyrophylum* namely (E) -N-Feruloyltyramine diacetate and and N-p-Coumaroyltyramine diacetate which had a mp 160-161^{o} and 160^{o} respectively (Sing et al., 1996). Other compounds from Piper has a mp varying between 58-305^{o} (Singh et al., 1996; Kuo et al., 2002; Banerji et al., 2002; Dharmaratne et al., 2002).

The 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 (Umoh et al., 2013). Our previous research found that 2:12 g of Pc-extract contained 12.0 mg Pc-1 and 12.1 mg Pc-2 (Harttini et al., 2014). The current study measured the activity of Pc-extract and isolates/pure compounds from its extract to determine the quantitative contribution of pure compounds on the activity of the extract. Mathematically, PP and PI of isolates with a dose of 5 mg/kg body weight would be equivalent to PP and PI generated by Pc-1 contained in 876 mg/kg body weight Pc-extract and Pc-2 contained in 883 mg/kg body weight Pc-extract. However, our current results showed that at a dose of 450 mg/kg body weight Pc-extract, or almost half of mathematical calculation, already indicates PP and PI which is equivalent to 5 mg/kg isolates. This indicates that there is a synergistic or additive effect that is responsible for higher PP and PI Pc-extract than isolates. The same condition was also found in compounds isolated from *P. methysticum* which showed anti-anxiolytic activity. The root Pc-extract showed higher anti-anxiolytic activity than each individual major compound (Umoh et al., 2013). Occasionally, extract the potential to produce active compounds be wasted because the activity was not detected during the fractionation. Failure to isolate active compounds from the extract may also be caused by instability of the active compound during the extraction (Deharo et al., 2011). Extracts, fractions, as well as isolated compounds from red betel leaves can increase the phagocytic activity of macrophages (Hartini et al., 2013; Kustiawan, 2012; Hartini et al., 2014), this indicated that the Pc-1 and PC-2 was relatively stable and the isolation methods applied to separate Pc-1 and pc-2 from the extract did not cause damage to the two compounds. The effect of increasing phagocytic activity of macrophages probably due to other compounds (other than the PC-1 and PC-2) in Pc-extract which has similar activity, resulting in an additive effect, or there are other compounds that are able to optimize the effects of Pc-1 and Pc-2 resulting in a synergy effect.

It was not recommended to use Pc-extract at the dose of 150 mg/kg body weight. At the dose of 150 mg/kg body weight Pc-extract, the NO production was increased significantly but there was no significant increasing occur on the other groups. Phagocytic cells play an important role in the immune system mechanism. However, when the phagocytic cells were over activated, the cells will be damaged through their Reactive Oxygen Species (ROS) and NO productions. In the phagolysosome process, inducible Nitric Oxide Synthase (iNOS) and ROS were activated. Nitric oxide is a product of arginin reaction catalyzed by iNOS enzyme. NO regulates inflammatory erythema and edema, and regulate the synthesis of inflammatory mediators and some inflammatory cell function. NO is synthesized in large quantities which are activated by macrophages as a vasodilator modulate vascular responses in acute inflammatory

reaction. Pc-extract is an immunostimulant, which is able to increase macrophages phagocytic activity known relatively high. As already mentioned, the dose of 300 and 450 mg/kg body weight did not increase NO production significantly. Similar with our previous results that the treatment of Pc-1 and Pc-2 produced low level of NO although the phagocytic activities were relatively high (Hartini et al., 2014). The condition may be due to the ability of the isolated compound and the extract to maintain the function of immune cells from the effect of macrophage phagocytic over activity. This indicates the activity of antioxidant and anti-inflammatory of the Pc-extract and its two compounds. The isolated compound is neolignan, a dimer of lignans one subgroup of polyphenols (non-flavonoid). One of the polyphenols as anti-inflammatory mechanism is to inhibit pro-inflammatory enzymes, such as cyclooxygenase (COX), lipooksigenase (LOX), and iNOS; through activation of the peroxisome proliferator activated receptor gamma (PPARγ). Polyphenols inhibit eicosanoid which form the enzyme phospholipase A2, cyclooxygenase, and lipooksigenase causes a decrease in the concentration of prostanoid and leukotriene (Santangelo et al., 2007). Anti-inflammatory activity of Piper species have also been reported. *P. longum* Linn fruit extract is reported to have anti-inflammatory activity (Kumar et al., 2009), as well neolignan derived from extracts of *P. nigrum* L. (Tasleem et al., 2014). The antioxidant and anti-inflammatory activity of both compounds and Pc-extracts need to be further studied.

Treatment with Pc-extract with the dose of 150, 300, and 450 mg/kg body weight, there is no difference in the histopathological features of mice liver in the treatment group compared to its control group. There is no change in histopathological features of the liver after treatment with Pc-1(showed with an arrow in Figure b). Degeneration Hydrophobic is a reversible processes. If the use of the substance is stopped, the condition of DH can be normal again Dancygier et al., 2010). Pc-2 at a dose of 5 mg/kg body weight was recommended to be used in the short-term treatment.

Apartate Transaminase (AST)/ Glutamic Oxaloacetic Transaminase (SGOT) and Alanine transaminase (ALT)/ Glutamic Pyruvic Transaminase (SGPT) serum are the enzymes produced by liver. Although many studies showed that the increase of hepatic enzymes serum was not a directly linked for liver injury, increase levels were responsible to cause inflammation, cellular leakage and damage of cell membrane to cells in the liver (Kausar et al., 2010). The results of the current study showed that mice AST treated with extract and isolated compounds were significantly lower than the negative control mice group, but the ALT value was not significantly different compared to the negative control group mice. In general, 5 mg/kg body weight Pc-1, 5 mg/kg body weight Pc-2, and Pc-extract of 150, 300, and

450 mg/kg body weight reduced the levels of AST. Further research is needed to investigate the hepatoprotective effect of extracts and isolated compounds. Acute toxicity testing is one of the parameter for the measurements of drug safety. Acute toxicity tests on mice showed that treatment with 15 mg/kg body weight piperine (isolated from *P. nigrum* L. extract) or 15 mg/kg body weight extract of *P. nigrum* L. mice resulted zero persen (%) mortality. A hundred % of mice mortality occurred in mice treated with the isolated compound and extract at a dose of 25 mg/kg body weight (Kumar et al., 2009). At doses of 450 mg/kg body weight Pc-extract did not cause the death of mice even there was no change in the liver and kidneys histopathological features, this indicates that Pc-extract safer than *P. nigrum* L. To compare the level of isolates safety of both species Piper, it is necessary to conduct further research to establish the LD_{50} of Pc-1 and Pc-2.

Conclusion

The 450 mg/kg body weight of Pc-extract had equal macrophage phagocytic activity compared to 5 mg/kg body weight of two neolignans (Pc-1 and Pc-2) isolated from Pc-extract. The 150 mg/kg body weight of Pc-extract had high production of NO than 300 and 450 mg/kg body weight and 5 mg/kg body weight Pc-1 and Pc-2. The treatment of 5 mg/kg body weight Pc-1 and 150, 300, and 450 mg/kg body weight Pc-extract treatment did not affect the histopathological features of mice liver. The treatment of 5 mg/kg body weight Pc-2 caused liver hydropic degeneration.

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Conflict of Interest: The authors declare no conflict of interests.

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Title of manuscript: The Comparison of Two Neolignans Isolated from Red Betel Leaf and Its Extract against Macrophage Phagocytic Activity, the Level of AST, and Histopathological Features of the Liver in Mice

Section	Line	The changes
Abstract	-	The abstract has been rewritten with emphasizing in the result, methods and final conclusion
Introduction	1 and 2	It has been added the information that Pc-1 and Pc-2 were isolated by Kustiawan
Material and method	-	It has been added the detail method of fractionation.
Material and method	-	It has been added the explanation that Pc-1 and Pc-2 were donated by Kustiawan
Material and method	-	The time of treatment of Pc-1, Pc-2, and Pc-extract on mice for 14 days were done according to Kaniwani et al., (2008)
Result (Isolated Compound)	-	It has been added the information that Pc-1 and Pc-2 were analysed in the Pc-extract using GC-MS and detected in the minute of 30.145 and 29.708 respectively
Result (Isolated Compound)	-	It has been added the GC-MS chromatogram of Pc-1, Pc-2, and *Piper crocatum* leaf extract (Pc-extract)
Conclusion	-	The conclusion has been rewritten

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