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Original Article

Down-regulation of complement genes in lipopolysaccharide-challenged zebrafish (*Danio rerio*) larvae exposed to Indonesian propolis

Regulação negativa dos genes do complemento em larvas de peixe-zebra (*Danio rerio*) desafiadas com lipopolissacarídeo expostas à própolis indonésia

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Abstract

Although propolis has been reported for having anti-inflammatory activities, its effects on complement system has not been much studied. This research was conducted to find out the effects of Indonesian propolis on the expression levels of *C3*, *C1r/s*, *Bf*, *MBL*, and *C6* in zebrafish larvae which were induced by lipopolysaccharide (LPS). Counting of macrophages migrating to yolk sac and liver histology were carried out. Larvae were divided into four groups: CON (cultured in E3 medium only), LPS (cultured in a medium containing 0.5 µg/L LPS), LPSIBU (cultured in a medium containing LPS, and then treated with 100 µg/L ibuprofen for 24 hours), and LPSPRO (cultured in a medium containing LPS, and then immersed in 14,000 µg/L propolis for 24 hours) groups. The results showed that complement gene expression in larvae from the LPSIBU and LPSPRO groups were generally lower than in larvae from the LPS group. The number of macrophage migrations to the yolk in the LPSPRO group was also lower than in the LPS group. Histological structure of liver in all groups were considered normal. This study shows that Indonesian propolis has the potential to be used as an alternative to the substitution of NSAIDs.

Keywords: Indonesian propolis, complement system, zebrafish.

Resumo

Embora a própolis tenha sido relatada por ter atividade anti-inflamatória, seus efeitos no sistema complemento, uma parte do sistema imunológico inato, não foram muito estudados. Esta pesquisa foi conduzida para descobrir os efeitos da própolis da Indonésia nos níveis de expressão de C3, C1r/s, Bf, MBL e C6 em larvas de peixe-zebra induzidas por lipopolissacarídeo (LPS). Foram realizadas contagens de macrófagos que migram para o saco vitelino e histologia do fígado. As larvas foram divididas em quatro grupos: CON (cultivadas apenas em meio E3), LPS (cultivadas em meio contendo 0,5 µg/L de LPS), LPSIBU (cultivadas em meio contendo LPS e, em seguida, tratadas com 100 µg/L de ibuprofeno por 24 horas) e LPSPRO (cultivado em meio contendo LPS, e então imerso em própolis 14,000 µg/L por 24 horas). Os resultados mostraram que a expressão do gene do complemento em larvas dos grupos LPSIBU e LPSPRO foi geralmente menor que em larvas do grupo LPS. O número de migrações de macrófagos para a gema no grupo LPSPRO também foi menor que no grupo LPS. A estrutura histológica do fígado em todos os grupos foi considerada normal. Este estudo mostra que a própolis indonésia tem potencial para ser utilizada como alternativa na substituição dos AINEs (anti-inflamatórios não esteroides).

Palavras-chave: Própolis Indonésia, sistema complementar, peixe-zebra.

1. Introduction

Inflammation is a well-known mark of microbial infections or injuries without any microbial infections (Medzhitov, 2008), characterized by swelling (*tumor*), redness (*rubor*), pain (*dolor*), and increased heat (*calor*) followed by the loss of function (*functio laesa*) (Markiewski and Lambris, 2007). However, understanding of the fundamental mechanisms that form the inflammatory response has been increasingly expanded in recent years. Complement system has been identified as a contributor to various inflammatory pathologies, including pathogen sensing and killing, damaged cell clearance, immune complex handling, and modulation of adaptive immune responses. In general, a controlled inflammatory response is beneficial since it can provide protection against infection. However, inadequate, excessive, or improperly regulated inflammation or complement activation can lead to a

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septic shock (Charchaflieh et al., 2012) that can lead to organ failure (Polat et al., 2017).

Complement is comprising of more than 50 proteins (Thorgersen et al., 2019). It can be activated through classical pathway, lectin pathway, and alternative pathway. These three pathways connect on the formation of C3 convertases (Markiewski and Lambris, 2007). Complement activation leads to opsonization process followed by phagocytosis, lysis, and inflammation (Dunkelberger and Song, 2010).

The classical pathway is activated when C1 complex, consisting of C1q and the proteases C1r and C1s, binds to pathogen-binding antibodies (Ricklin and Lambris, 2013). The lectin pathway is activated when mannose-binding lectins (MBL) or ficolins recognize conserved pathogenic carbohydrate motifs, activating the MBL-associated serine proteases (MASPs) (Dunkelberger and Song, 2010). Through activation of C2 and C4, both pathways lead to the assembly of C3 convertase complexes, which cleave the abundant plasma protein C3 into an anaphylatoxin fragment (C3a) and the opsonin C3b (Ricklin and Lambris, 2013). The alternative pathway is activated when C3 forms the initial C3 convertase through hydrolysis, in the presence of Factor B and Factor D, leading to additional C3 cleavage and formation of the C3 convertase and C5 convertase (Markiewski and Lambris, 2007). Those pathways will result in the formation of the complement system effectors: anaphylatoxins, the membrane attack complex (MAC), and opsonins (Ricklin and Lambris, 2013).

Immune cell migration is also an important mechanism for the inflammatory response. Macrophages are one of the immune cells that react most quickly to pathogens and also have a longer lifespan compared to neutrophils (Galli et al., 2011). The macrophages that are activated due to the presence of pathogens will increase the production of proinflammatory cytokines, such as TNF-α, IL-1β, and IL-6, so that they can attract other inflammatory cells to migrate to the site of infection and/or inflammation (Kim et al., 2019).

Hepatocytes, the major parenchymal cells in the liver, synthesize 90% of complement proteins in serum involved in the classical pathway (C1r/s, C2, and C4), alternative pathway (C3 and Factor B/*Bf*), lectin pathway (MBL, MASP1-3, and Map19), terminal components (C5, C6, C8, and C9), and regulators (factors I, H, and C1 inhibitors) (Qin and Gao, 2006). Hepatocytes also have important roles in metabolism and detoxification (Zhou et al., 2016). Although the ability to regenerate is relatively high, after acute injury or resection, this regenerative ability is overwhelmed by two major scenarios: severe acute liver injury or severe chronic liver injury (Forbes and Newsome, 2016). Thus, understanding the mechanism of the liver disease is very important for the development of treatment (Wilkins and Pack, 2013).

NSAIDs (non-steroidal anti-inflammatory drugs) such as ibuprofen, aspirin, and paracetamol are commonly used to alleviate pain, fever, and inflammation. Unfortunately, these anti-inflammatory drugs have negative side-effects if they are used for a long time in high doses. Some serious effects possibly resulting from NSAIDs consumption are the increasing risks of heart attack, stroke, peptic ulcer, bleeding in the digestive system, and kidney disease (Wehling, 2014). Therefore, it is important to explore new therapeutic methods for inflammation with fewer side effects.

Propolis has been reported to possess some biological activities which are antibacterial, antiviral, antifungal, wound healing, and anti-inflammatory (Oryan et al., 2018). Chemical contents in propolis vary depending on geographic location, plant source, season, and bee species (Huang et al., 2014). The Indonesian propolis used in this study is produced by stingless bees *Trigona* sp. from Subang, West Java. This research was conducted to find out the effects of Indonesian propolis on the expression levels of *C3* (central component), *C1r/s* (classical pathway), *Bf* (alternative pathway), *MBL* (lectin pathway), and *C6* (terminal component) in zebrafish larvae which were induced by lipopolysaccharide (LPS), a major component of the outer membrane of Gram-negative bacteria, that can stimulate innate immune system (Yücel et al., 2017).

2. Materials and methods

2.1. Propolis extraction

The propolis used in this study originates from Subang, West Java, and is produced by stingless bees *Trigona* sp. The raw propolis was extracted using ethanol, and its chemical contents were analyzed using GC-MS (Gas Chromatography-Mass Spectrometry).

2.2. Zebrafish Maintenance

Zebrafish were maintained in accordance with standard protocol (Westerfield, 2000). Zebrafish embryos and larvae were obtained from natural spawning and kept in Petri dish containing E3 medium (5 mM of NaCl, 0.17 mM of KCl, 0.33 mM of $CaCl_2$, 0.33 mM of $MgSO_4$, without methylene blue) at 26-30°C (Nusslein-Volhard and Dahm, 2002).

2.3. Pigment removal

Pigment removal was carried out on the larvae whose macrophage migrations would be calculated. Pigment removal was performed by adding 0.2 mM PTU (N-phenylthiourea) to the medium at the end of gastrulation (about 10 hpf) (Karlsson et al., 2001). The medium containing PTU was replaced daily.

2.4. Treatment of zebrafish larvae

Treatment of zebrafish larvae was carried out by immersion in 6-well plates. Thirty 5-dpf-larvae per group were placed in well containing 6 mL of E3 medium. Larvae were divided into four groups: control, negative control (LPS) group, positive control (LPSIBU) group, and LPSPRO group. The control group consisted of larvae which were cultured in E3 medium only. The LPS group comprised of larvae which were cultured in a medium containing 0.5 µg/L LPS (Sigma-Aldrich). The LPSIBU group encompassed larvae which were cultured in a medium containing LPS, and then treated with 100 µg/L ibuprofen for 24 hours by the immersion method (Jones et al., 2012). The LPSPRO group comprised of larvae which were cultured

in a medium containing LPS, and then given 14,000 µg/L propolis treatment for 24 hours by the immersion method.

2.5. Quantitative RT-PCR for expression level analysis

Total RNA was isolated from euthanized larvae (30 larvae per sample) using the PureLink™ RNA Mini Kit (Thermo Fisher Scientific). The existence of RNAs was analyzed using 2% agarose gel electrophoresis. The parameters of the RNAs existence were visualized by the presence of 28S (4.8 kb) and 18S (2.0 kb) ribosomal RNAs (Aranda et al., 2012).

Before cDNA was synthesized, treatment was carried out using DNAse I (Thermo Fisher Scientific) to remove DNA contamination. A total of 1 µg RNA from each sample was mixed with 1 µL 10× reaction buffer, 1 µL DNAse I, and nuclease-free water, until the final volume was 10 µL. The mixture was incubated at 37°C for 30 minutes and followed by DNAse I inactivation by adding 1 µL of 50 mM EDTA. After that, the mixture was incubated at 65°C for 10 minutes.

The cDNA synthesis was carried out using SensiFAST™ cDNA Synthesis Kit (Bioline). A total of 11 µL RNA samples which were treated using DNAse I was mixed with 4 µL 5× buffer first strand, 1 µL reverse transcriptase, and nuclease-free water, until the final volume was 20 µL. The mixture was then incubated using T100™ Thermal Cycler (Bio-Rad) at 25°C for 10 minutes (primers annealing), 42°C for 15 minutes (reverse transcription), and 85°C for 5 minutes (reverse transcriptase inactivation). The resulting cDNA was stored at -80°C for further use.

Real-time PCR was carried out using T100™ Thermal Cycler (Bio-Rad) with a total volume of 10 µl containing specific gene primers, cDNA, and GoTaq® Green Master Mix (Promega). cDNA products were denatured for 3 minutes at 95°C, amplified with 34 cycles (with 30 seconds denaturation at 95°C, 30 seconds annealing at the temperatures shown in Table 1, and 30 seconds extension at 72°C), and 5 minutes final extension at 72°C. β-actin was used as a reference for internal standardization. The gene-specific primer sequences used are mentioned in Table 1 (obtained from Genetika Science Jakarta, Indonesia) based on study conducted by Wang et al. (2008). Relative gene expression analysis was carried out using method conducted by Livak and Schmittgen (2001).

2.6. Neutral red staining

The treated larvae whose pigments had been removed were stained with a neutral red of 2.5 µg/mL concentration for 6-8 hours in the dark (Yang et al., 2014). Macrophages and lysosomes coalesced to form large red aggregates, so that they were easy to detect in the Zebrafish larvae whose pigments had been removed. The macrophages migration to the yolk sac was observed under a microscope.

2.7. Liver histology

Euthanized larvae were fixed in freshly prepared Bouin's solution at room temperature for 24 hours, dehydrated in alcohol series, embedded in molten paraffin, and sagittally cut at 5 µm of width. Larval sections were stained using hematoxylin-eosin (HE).

3. Results

3.1. Chemical analysis

Propolis sample used in this research was the same as that used in Wibowo et al. (2020). The CG-MS chemical analysis revealed 25 different compounds. Most of these compounds are phenolic compounds with recognized therapeutic properties. The most abundant compound was m-guaiacol (phenol), 14.07%, as mentioned in Wibowo et al. (2020).

3.2. Expression levels of complement genes

The highest C3 expression level was in larvae from the LPS group (as shown in Figure 1.A). This indicates that more C3 is needed by the larvae from the LPS group to

Table 1. Oligonucleotide Primers Sequence.

Name		Sequence	Dissociation temperature of the primer/T_m (°C)	Accession Number
β-actin	F	TTCACCACCACAGCCGAAAGA	56.52	AF057040
	R	TACCGCAAGATTCCATACCCA	55.07	
C3	F	GTATTACTCACCCGATGCCCG	56.92	AB235997.1
	R	AGATGGGGTTCACAGGCTTTAAT	55.91	
C1r/s	F	GAGTTGTGTTTCAGATGGCTTGC	56.96	XM684254.2
	R	CATTGCGATGGTCTTCAGTTCC	56.12	
Bf	F	GCTGTCCACGGAAAATAAGG	53.27	NM_131338
	R	TCGGTCGCATCTGCCACT	58.07	
MBL	F	GCAGAGCCAGGAGTGAATGTG	57.57	AF227738.1
	R	ACCTTCTCAATCAGGGCAATC	54.22	
C6	F	ATGACGCTGGCAAGGAAACT	56.62	BC057429
	R	TGTCTGAACCGCAGGGCTC	58.77	

activate the complement system, so that it can eliminate the LPS. This phenomenon is contrary to the larvae from the LPSIBU and LPSPRO groups which showed a decrease in C3 gene expression because the inflammation can be suppressed by ibuprofen and the Indonesian propolis. Thus, further complement activation is not required. The result is consistent with Georgieva et al. (1997) who suggested that propolis can generate inactivation of the C3 complement, so that further complement activity, including inflammation, can be inhibited.

In larvae from the LPSIBU and LPSPRO groups, there were decrease in the relative gene expression of the C1r/s compared to LPS group (see Figure 1.B). This result is in line with the study of Dimov et al. (1992) that propolis can decrease C1 gene expression and Ivanovska et al. (1995) that propolis can inhibit the complement in the classical and alternative pathways. In zebrafish larvae, the adaptive immune system has not been functional yet, so that the activation of the complement system in the classical pathway is still not yet competent. This is in accordance with a research conducted by Wang et al. (2008).

Relative expression level of *Bf* in larvae from the CON group was almost the same as in larvae from the LPS group (see Figure 1.C). Alternative pathway is also activated under normal conditions to maintain tissue homeostasis with very low level of activation (Merle et al., 2015). In the LPSPRO, a decrease in the relative expression level of *Bf* was detected. This finding is in accordance with the research conducted by Ivanovska et al. (1995) which shows that propolis can inhibit the complement in the classical and alternative pathways.

The expression levels of *MBL* in all groups were not significantly different. It indicates that the *MBL*, as a part of the lectin pathway, in 5 dpf larvae was still not competent yet to be activated. The result is in accordance with the study results of Wang et al. (2008) that LPS challenge did not trigger significant change in *MBL* expression until 17 dpf. In addition, treatments using ibuprofen and the Indonesian propolis do not have significant effects on the *MBL* expression.

Relative expression level of *C6* in the LPSPRO group showed a decrease compared to the LPS group (see Figure 2). It indicates that the Indonesian propolis can inhibit the terminal activity of the complement system. Since the expression level of C3 had been suppressed, the expression of other complements which are involved in the terminal activity, especially *C6*, which plays a role in the formation of MAC pores and targeted lysis, could be inhibited.

3.3. Macrophage migration

In larvae from the CON group, no macrophages migration to the yolk sac was observed (as seen in Figure 3). It was due to the absence of pathogenic molecules (LPS) which can cause inflammatory response, activate macrophages, and attract them to the yolk sac. There was a higher number of macrophages in the LPS group than those in the CON, LPSIBU, and LPSPRO groups.

3.4. Histological structure of livers

Based on the observation, the liver histological structures of all groups were considered normal (see Figure 4). The cell nuclei were clearly visible and the hepatocytes were normally arranged. Changes that occur on the hepatocytes by the LPS are only affected by the dose; the higher the dose of LPS, the more the liver is affected (Bates et al., 2007). The LPS given in this study had sublethal doses. Thus, its effects

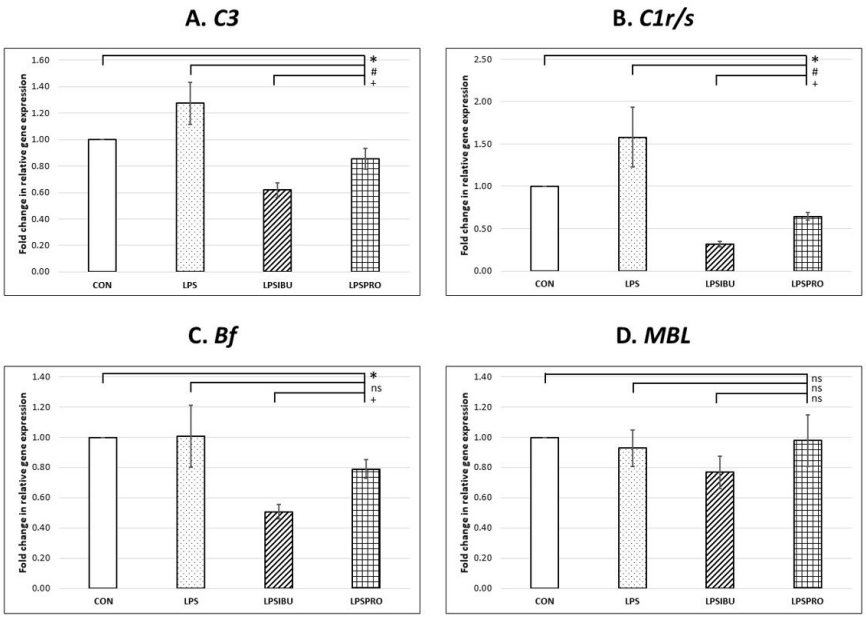

Figure 1. Relative expression levels of *C3* (A), *C1r/s* (B), and *Bf* (C) in larvae from the LPSIBU and LPSPRO groups were lower than in larvae from the LPS group ($P<0.05$); relative expression levels of *MBL* (D) in all group were not different. Data are expressed as mean ± SEM. Significances marked with *, #, and + refer to comparisons to the CON, LPS, and LPSIBU group, respectively. *,#,+ $P < 0.05$; ns, not significant.

on liver structure were not severe. In addition, zebrafish have IAP (intestinal alkaline phosphatase) produced by intestinal epithelium, which could dephosphorylate several serotypes from the LPS, so that the non-toxic products will be produced (Bates et al., 2007).

4. Discussion

C3 protein is the central constituent of the complement system since all of the activation pathways converge on the formation of the C3 convertases (Ricklin and Lambris, 2013). The complement anaphylatoxin resulted can then attract immune cells, including macrophages at the sites of infection, among which is the yolk sac. This is one of the factors that can initiate an increasing number of macrophage migrations to the yolk sac in larvae from the LPS groups. In the LPSIBU and LPSPRO groups, C3 gene expression levels were lower than in larvae from the LPS group. Thus, the number of macrophage migrations to the yolk sac decreased. This result is consistent with another study conducted by Ansorge et al. (2003) shows that propolis can reduce DNA synthesis in PBMC (peripheral blood mononuclear cells), including monocytes. Propolis can also suppress the production of proinflammatory cytokines (IL-1β, IL-2, IL-4, and IL-12) in macrophages.

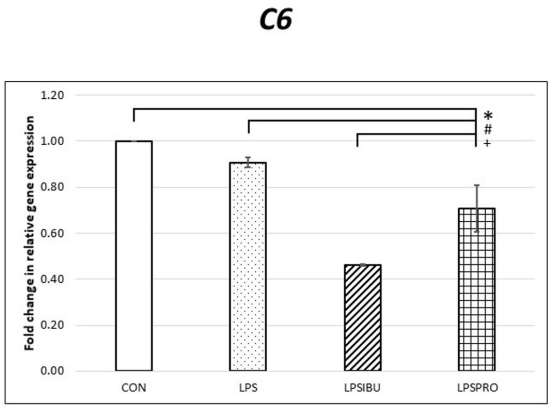

Figure 2. Relative expression level of *C6* in larvae from LPSPRO group was lower than from LPS group (P<0.05). Data are expressed as mean ± SEM. Significances marked with *, #, and + refer to comparisons to the CON, LPS, and LPSIBU group, respectively. *,#,+ $P < 0.05$.

Macrophages can easily recognize the LPS using TLR4 receptors (Fang et al., 2017). In addition to receptors for recognizing the LPS, macrophages also have other receptors for recognizing complement, which are C3aR and C5aR receptors for recognizing complement anaphylatoxins C3a and C5a; and CR1, CR3, CR4, and CRIg receptors for recognizing C3b as opsonin for pathogens and other foreign agents, so that the phagocytosis can be carried out (Bohlson et al., 2014). Complement anaphylatoxins, C3a and C5a, are chemoattractants that are capable to recruit immune cells, such as neutrophils, monocytes, and eosinophils, so that they can activate phagocytic cells, induce granular secretion, and increase oxidation formation (Guo and Ward, 2005; Luo et al., 2019).

In larvae from the LPSIBU and LPSPRO groups, there were no changes in the liver histological structure. According to a research conducted Traversa et al. (2003), ibuprofen has low toxic level to the liver because it has a short half-life in plasma, so that it does not form pathological metabolites. The liver treated using the Indonesian propolis showed no changes, as in the other groups. Propolis is known for having various biological activities, among which are hepatoprotective (Miguel and Antunes, 2011). A research conducted by Kismet et al. (2017) shows that propolis has positive effects on the histopathology of NAFLD (non-alcoholic fatty liver disease) because propolis has anti-inflammatory effects. This shows that anti-inflammatory ability is very important in maintaining liver homeostasis from damage possibly caused by inflammation.

Indonesian propolis used in this research contained 14.07% of m-guaiacol (phenol) and other phenolic compounds (Wibowo et al., 2020). Phenolic compounds are the secondary metabolites which present in plants as a defense mechanism against ultraviolet

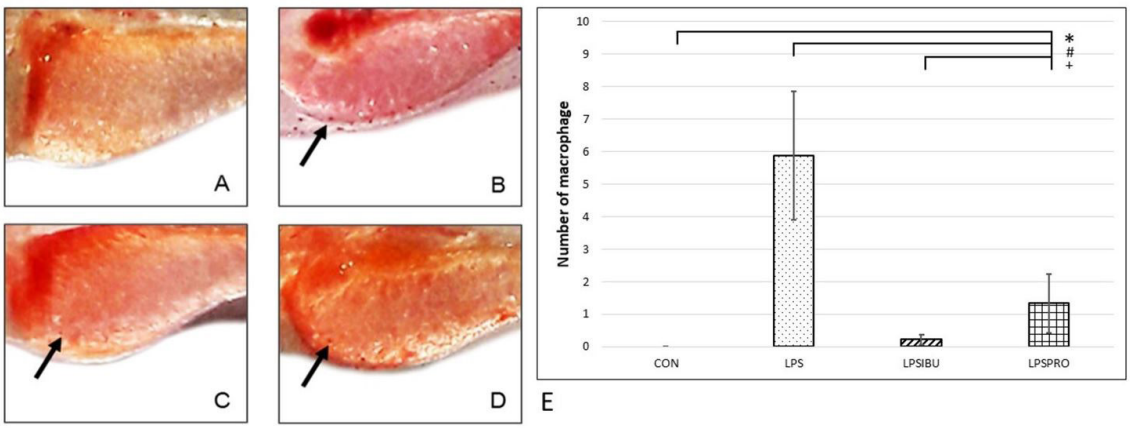

Figure 3. The number of macrophage migration in LPSPRO group was lower than in LPS group (P<0.05). The macrophages were seen as red dots (see arrows): A (CON); B (LPS); C (LPSIBU); D (LPSPRO). E. Quantification result. Data are expressed as mean ± SEM. Significances marked with *, #, and + refer to comparisons to the CON, LPS, and LPSIBU group, respectively. *,#,+ $P < 0.05$.

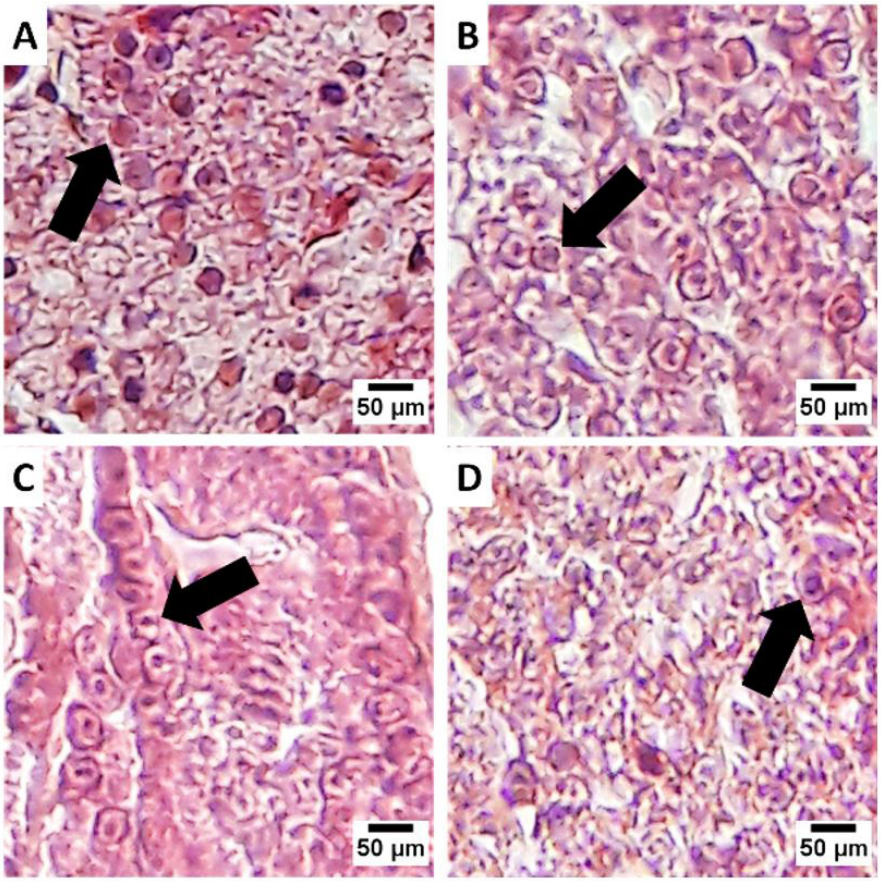

Figure 4. Histological structure of the livers, A (CON); B (LPS); C (LPSIBU); D (LPSPRO). Nuclei are pointed by the arrows.

radiation or pathogens (Pandey and Rizvi, 2009). Those compounds are presumed to exert anti-inflammatory effects (González et al., 2011). A research conducted by Murakami et al. (2007) shows that guaiacol can inhibit COX-2 (cyclooxygenase-2) and NF-κB transcription factors on macrophages which are induced by the LPS. COX-2 is an enzyme that can convert arachidonic acid into prostaglandins (PGE2). Prostaglandins can escalate increased vascular permeability, causing edema, pain, and fever when an inflammatory response occurs (Dennis and Norris, 2015). Ibuprofen also works by inhibiting COX-2, so that it can relieve inflammation, pain, and fever due to the inflammatory response (Bushra and Aslam, 2010). NF-κB transcription factors stimulate the formation of proinflammatory cytokines such as TNF-α and IL-1β (Tsubaki et al., 2015).

5. Conclusion

In summary, this study shows that the Indonesian propolis reduced the complement genes expression level, the number of macrophage migrations to the yolk, and cause no changes in liver structure. These results suggest that Indonesian propolis has the potential to be used as an alternative to the substitution of NSAIDs (non-steroidal anti-inflammatory drugs). Further research on the mechanism is still necessary.

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