ETHANOL EXTRACT OF ARECA NUTS WAS ABLE TO IMPROVE THE HISTOPATHOLOGICAL FEATURES OF P. BERGHEI-INDUCED LIVER DAMAGE IN MICE

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Abstract: Adequate therapy is necessary to prevent further damage to the liver infected with Plasmodium sp. *Areca catechu and curcumin have the potential for malaria therapy, and scientific evidence is required to examine such potential either alone or in combination. This experimental study used a posttest-only group design involving 24 male Swiss mice (Mus musculus) as the subjects divided into 6 groups (@4 mice). P. berghei was injected intraperitoneally in 5 groups, and different types of treatment (4 days, feeding tube) were administered to 4 groups (K1 = chloroquine, K3 = ethanol extract of Areca nuts + curcumin, K4 = ethanol extract of Areca nuts, K5 = curcumin). The doses were 0.012mg/kgBW of chloroquine, 150mg/kgBW of Areca nut ethanol extract, and 30mg/kgBW of curcumin. K2 was the unhealthy group (infected with P. berghei without therapy), while K6 was the normal/healthy group. Parasitemia was examined in 3 days after induction by P. berghei (inclusion criterion: parasitemia >5%, exclusion criterion: parasitemia >15%). The liver was embedded in paraffin blocks and stained with HE. Observations were made to identify the presence of necrosis, portal inflammation, and hemosiderin. The data of histopathological changes in the liver was expressed in percentages. The administration of Areca nut ethanol extract was able to provide better histopathological features than curcumin therapy alone, in combination, or chloroquine therapy (K4, no necrosis; mild portal inflammation = 50%, moderate = 25%; hemosiderin = 25%). Areca nut ethanol extract had yet to show histopathological features that resembled a healthy condition (K6 = normal inflammation, mild, moderate = 50%, 50%, 0%, respectively; hemosiderin = 0%). The ethanol extract of Areca nuts alone was shortly able to improve the histopathological features of P. berghei-induced liver damage in mice.*

Keywords*: Areca nuts, Plasmodium berghei, histopathology of liver*

INTRODUCTION

Plasmodium sp. can cause organ damage. Research shows that hepatic abnormalities due to *Plasmodium* sp. infection are associated with deterioration in malaria, such as the incidents of cerebral malaria, shock, respiratory failure, and acute kidney failure.[1] Such deterioration can lead to the death of malaria patients. Liver failure will also increase the fatality rate of malaria patients.[2] Liver damage identification and adequate therapy for *Plasmodium* sp. infection are therefore required to prevent further complications or death.

In experimental animals, malaria can be induced using *P. berghei*. Compared with the other two types of *Plasmodium* (*P. chabaudi* and *P. yoelii*), induction by *P. berghei* can result in higher levels of parasitemia and hemozoin as well as more severe organ damage.[1,3] In the liver of experimental animals, *Plasmodium* sp. infection is characterized by the presence of focal necrosis, cholestasis, congestion, hemozoin deposition, and inflammation.[1,4] One type of pathogenesis which causes such morphological changes is oxidative stress. *P. berghei* infection increases free radicals and reduces antioxidant and glutathione levels.[1] Malaria therapy on the basis of the pathomechanisms of oxidative stress is potential to be developed as alternative therapy to treat malaria with resistance to standard therapy.

Areca catechu is an herbal plant with the potential to be an antimalarial drug candidate. The potential of *Areca catechu* extract has been identified in a number of in vivo and in vitro studies. The administration of butanol extract of *Areca catechu* leaves for 4 days at a dose of 150 mg/kg/day shows fair inhibition test results (39.1%, parasitemia growth inhibition) and a better survival rate (60%) compared to the control group.[5] The butanol fraction of *Areca nut* extract is highly potent for *P. falciparum* infection with 18µg/ml LC50.[6] *Areca nuts* extracted with 95% ethanol solvent shows a strong antioxidant activity (3.5 µg/ml).[7] These high antioxidant levels in ethanol extraction indicate the potential of *Areca nuts* to treat a disease whose etiopathology is associated with reduced antioxidants, such as in *P. berghei* infection.

Meanwhile, curcumin is a hepatoprotective and therapeutic drug substance. The administration of curcumin for 9 days prior to induction by alcohol can prevent hepatocyte damage.[8] Curcumin also shows potent antimalarial effects in both in vivo (IC50 = 4.34+1.59 µM and SI = 28.98) and in vitro studies (lowering the Glycogen synthase kinase-3β/GSK3β, a protein kinase enzyme involved in various disease and cellular processes). At a dose of 30mg/kgBW, curcumin can prophylactically and therapeutically inhibit GSK3β. Curcumin therapy reduces pro-inflammatory cytokines (TNF-α, IFN-gamma) and increases anti-inflammatory cytokines, including IL-10 and IL-4.[9] Such scientific evidence, which indicates the potential of *Areca catechu* and curcumin in animal models of malaria, has attracted the researchers' attention to further investigate the potential. In an animal model of *P. berghei*-induced malaria, the researchers used *Areca nuts* extracted in 95% alcohol and applied it alone or in combination with curcumin in accordance with the doses used in a previous study. The research is expected to add scientific evidence of *Areca catechu* and curcumin as potential malaria therapy.

RESEARCH METHODS

This study involved 24 male Swiss mice (*Mus musculus*) obtained from UD Wistar Yogyakarta, an experimental animal farming. The inclusion criteria in this study were male Swiss mice aged 8-10 weeks, weighing 20-30 grams, being physically healthy, and experiencing parasitemia >5% after being induced by *P. berghei*. Mice with physical and/or behavioral changes which indicated a disease and mice with

>15% parasitemia were excluded from this study.

After 7-day acclimatization, the 24 mice were divided into 6 groups (4 mice each). Parasitemia was induced in 5 groups (K1-K5). Therapy was performed after the experimental animals experienced parasitemia based on the inclusion criteria. Standard therapy (chloroquine) was administered to group K1. Different types of herbal therapy, both single and in combination, were administered to group K3-K5 (K3 = combination of Areca nut ethanol extract and curcumin, K4 = ethanol extract of Areca nuts, K5 = curcumin). Group K2 became the unhealthy control group (parasitemia without therapy), while group K6 was treated as the healthy control group (not induced by *P. berghei* and not receiving therapy).

Parasitemia was induced by intraperitoneally (i.p) injecting a single dose of *P. berghei* ($1x10^6$ parasites per 0.2 ml of blood, erythrocytic stage) into the experimental animals. Parasitemia condition was determined 3 days after induction by identifying the results of peripheral blood smears. The blood for parasitemia examination was obtained by making an incision in the lateral vein area (approximately 0.2 - 2 cm from the base of the tail). The obtained blood was then made into a thin blood smear and stained with 10% giemsa. The percentage of parasitemia was identified by counting the number of infected erythrocytes out of 5000 observed erythrocytes.

The *Areca nuts* used in this study were the nuts of young *Areca* fruit. Before the extraction, the determination of Areca nuts and fruit was performed at the pharmaceutical laboratory of Universitas Gadjah Mada. The *Areca nuts* used in this study were collected in Yogyakarta city. The *Areca nuts* were cut into small pieces and washed clean in running water. To remove moisture content, the *Areca nuts* were dried in an oven at a temperature of 30^oC – 50^oC and then ground in a blender to form powder. The ethanol extract of *Areca nuts* was prepared by adding 500 ml of 95% ethanol to *Areca nut* powder in a container covered with aluminum foil for 24 hours. After 24 hours, the dregs were sieved and the same amount of 95% ethanol was added (500 ml) and left for 12 hours. The procedure was replicated twice and the sieved product was then put into a water bath to obtain a viscous extract of *Areca nuts* (brown with typical odor of *Areca nuts*).

Different types of therapy were given to the experimental animals for 4 days. The administered dose of *Areca nut* ethanol extract was 150 mg/kgBW [5,10] while the curcumin was administered at a dose of 30 mg/kgBW.[9] All of the therapy types (single or combination) were administered once a day. The curcumin used in this study was in a powder form. The standard drug therapy was chloroquine at a dose of 0.012 mg/kgBW (ground into powder). The administered curcumin and chloroquine were dissolved in distilled water, while the ethanol extract of *Areca nuts* was dissolved in 10% DMSO. For group K3 which received combination therapy, the ethanol extract of *Areca nuts* and curcumin were administered alternately at a predetermined time.

Termination was performed on the 5^{th} therapy day (8 days after induction by *P. berghei*). The mice were anesthetized intramuscularly with Zoletil® injection at a dose of 0.03-0.05 ml. After the mice fell asleep, decapitation and necropsy were carried out to extract the liver. Liver fixation was performed by means of immersion technique with 10% formalin phosphate buffer saline (PBS) for 24 hours. The liver lobes were homogeneous among each of the mice. The liver tissue was then prepared in paraffin blocks, cut to a thickness of 5 µm, and stained with Hematoxylin Eosin (HE).

The histopathological changes to be observed in this study included the presence of hemosiderin deposition, portal inflammation, and hepatocyte necrosis. The grading methods for necrosis and portal

inflammation were adapted from Siegmund et al., (2002).[11] The parameter for hemosiderin was stated qualitatively (present or absent) while the other two parameters were stated semi-quantitatively (normal, mild, moderate, and severe). A normal condition would be shown by the absence of disorders/abnormalities. The criteria for necrosis were determined by comparing the percentage of the total area of necrosis to that of the liver parenchyma (mild = <10%, moderate = 10-25%, severe = 50%). The degree of portal inflammation was categorized by identifying inflammatory cell influx in the periportal area (mild = <1/3 portal tract, moderate = ½ portal tract, severe = >2/3 portal tract). Inflammation was characterized by the presence of lymphocytes, plasma cells, and macrophages while necrosis was identified from changes in the nucleus (shrinkage or pyknosis) and cell cytoplasm (the area with necrosis seemed more pale/eosinophilic).

Observations were made in single blinding by an independent pathologist from an agency unaffiliated with the researchers. A light microscope (Olympus CX2) was used for the observation in 5 fields of view with a 100x magnification. If abnormalities were found in the liver parenchyma, the morphological changes would be observed with a magnification of 400x.

Data from the three main parameters of observation in this study (hemosiderin, portal inflammation, and hepatocyte necrosis data) were stated as a percentage (the number of positive individuals divided by the total number of individuals in the group x 100%) and presented descriptively.

RESULTS AND DISCUSSION

This study shows that induction by *Plasmodium berghei* can lead to histopathological changes in the mouse liver. Such changes include hemosiderin accumulation, inflammation in the portal area, and necrosis (Figure 1). The administration of *Areca nut* ethanol extract is able to improve the histopathological features compared to either single curcumin therapy or combination therapy (K4= no necrosis; mild portal inflammation = 50%, moderate = 25%; hemosiderin = 25%). The combination therapy indicates similar potential with the single therapy of *Areca nut* ethanol extract in terms of the inflammation and necrosis parameters. Compared with the standard therapy group, the *Areca nut* ethanol extract therapy also results in better suppression of portal inflammation (K1 = mild, moderate inflammation at 75%, 25% respectively). However, the *Areca nut* ethanol extract therapy has not been able to show histopathological feature resemblance to that of healthy condition (K6 = normal, mild, moderate inflammation at 50%, 50%, 0% respectively; hemosiderin = 0%). The data of histopathological observation of the liver is presented in Table 1.

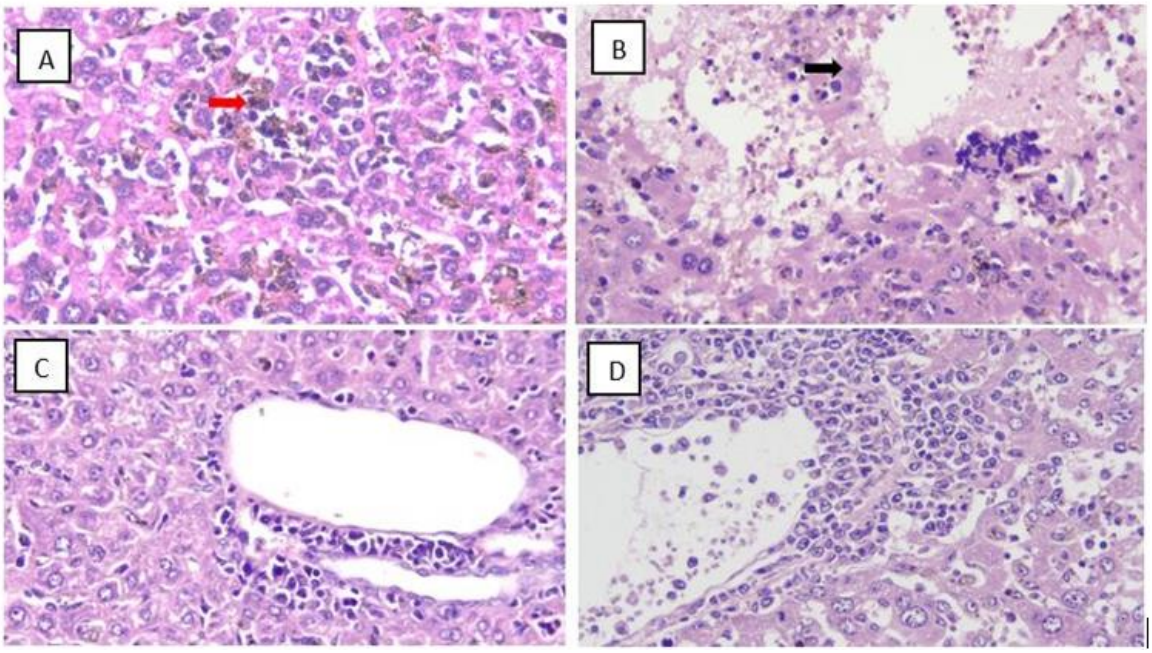

Figure 1. *P. berghei* infection causing hemosiderin deposition, inflammation, and necrosis (400x magnification, HE). A: Hemosiderin deposition indicated by a brownish color (red arrow), B: Mild necrosis (black arrow), C: mild portal inflammation, D; moderate portal inflammation

Table 1. Percentage of necrosis, portal inflammation, and hemosiderin in the mouse liver

Group	Necrosis (%)				Portal Inflammation (%)				Hemosiderin
(N)	Normal	Mild	Moderate	Severe	Normal	Mild	Moderate	Severe	(%)
K1 (4)	100	0	0	0	0	75	25	0	50
K2 (4)	50	50	0	0	25	75	0	0	50
K3 (4)	100	0	0	0	25	50	25	0	75
K4 (4)	100	0	0	0	25	50	25	0	25
K5 (4)	75	25	0	0	25	75	0	0	50
K6 (4)	100	0	0	0	50	50	0	0	0

Note: K1 = positive control group (parasitemia + chloroquine therapy), K2 = parasitemia without therapy, K3 = parasitemia + combination therapy (*Areca nut* ethanol extract + curcumin), K4 = parasitemia + *Areca nut* ethanol extract, K5 = parasitemia + curcumin therapy, K6 = healthy group

The results of this study indicate that the administration of *Areca nut* ethanol extract for a short term (4 days) can result in better histopathological features compared to the combination therapy, curcumin therapy, and standard therapy. *Areca nut* ethanol extract therapy, however, has not been able to provide histopathological features that resemble those of the liver of the healthy group. Hemosiderin deposition in the liver remains observable in all types of the therapy given in this study, whereas the portal inflammation is a histological feature which can also be found in healthy individuals.

The use of *Areca catechu* as herbal medicine remains to have pros and cons. A large number of studies have proved that Areca catechu has therapeutic effects [6,12,13], but some other studies have demonstrated that therapy involving *Areca catechu* results in negative or even carcinogenic effects. [14–16] This indicates that research to

examine the therapeutic dose of *Areca catechu* as herbal medicine should continue to be further developed.

Some research shows that *Areca nuts* has therapeutic effect. *Areca nut* extract is known to have antibacterial and antiviral effects.[6] Extract of *Areca catechu* also has the potential to support wound healing.[12] Other research has also shown that *Areca nut* extract is potential against malaria. In line with these findings, the therapy using *Areca nut* extract at a dose of 150 mg/kgBW for 4 days in this study shows the potential for hepatic tissue repair in *P. berghei*-induced animals (minimal hemosiderin deposition without necrosis). However, portal inflammation can still be found in the therapy using *Areca nut* extract (50% mild, 25% moderate). Overall, such potential is considerably better than therapy using chloroquine. No research has reported the therapeutic potential of *Areca nut* extract and curcumin combination. In this study, such combination can only suppress the presence of necrosis but is unable to treat hemosiderin and portal inflammation. This indicates that the therapeutic potential of *Areca nut* extract at a dose of 150 mg/kgBW against malaria is achieved as single therapy. The use of *Areca nuts* is also believed to have low toxicity. A study shows that the administration of the simplicia powder of *Areca nuts* macerated using 95% ethanol to rats for 3 weeks results in no toxic effects on the kidneys and liver based on functional and morphological analyses.[17]

When entering the body, *Plasmodium* induces inflammation which is a form of the host's response to eliminate invading pathogens. In malaria, inflammation is unable to eliminate the *Plasmodium* that enters the body and adversely causes severe damage to the host. The activation of macrophages and neutrophils during an inflammatory process in malaria results in increased ROS levels and increased pro-inflammatory cytokines. Oxidative stress can raise cell fragility (thus raising hemolysis and thrombocytopenia) while pro-inflammatory cytokines induce receptor formation in the endothelium to recognize *plasmodium*-infected erythrocytes.[18,19] The process by which the receptors recognize erythrocytes will result in cytoadherence that can eventually lead to tissue ischemia.[19] Such ischemic condition can deteriorate cell conditions during which the cells also experience inflammation due to malaria infection. When the cells are unable to survive this condition, they end up as necrosis.

A study found that parasitemia levels in malaria infection negatively correlate with antioxidant levels, thus suggesting that the higher the level of parasitemia, the lower the antioxidant level. In a malaria infection with 5-10% parasitemia, the GSH levels will decrease by 20%. Meanwhile, in 20-30% parasitemia, the decrease in GSH reaches 45% [3]. Reduced antioxidant levels can result in hemolysis and tissue damage.[1,20] Similar to such findings, this study shows that 5-12% parasitemia leads to portal inflammation, necrosis, and hemosiderin deposition. However, the liver damage in this study is relatively mild (50% necrosis and mild portal inflammation in the unhealthy control group).

Hemosiderin accumulation in the liver indicates deposition of erythrocyte destruction in hepatocytes or macrophages.[21] Hemolysis leads to heme and globin formation. Heme will experience deposition in the form of hemosiderin or ferritin.[19] Hemosiderin accumulation can be observed in the liver of *plasmodium*-infected animals.[21] Similarly, this study found that 50% of the *P. berghei*-infected animals exhibits hemosiderin deposition.

Areca nuts contain strong antioxidants which can be expected to overcome liver damage.[7,22] No studies have shown the potential of *Areca nuts* to improve liver damage, particularly in malaria conditions. This study reveals that the use of *Areca nut* extract alone can reduce damage to the liver. When used in combination with curcumin, Areca nut extract has been

unable to provide a therapeutic effect on the liver of *P. berghei*-infected mice. Although research by Ali, et al.[9] indicates that curcumin has the ability to reduce different pro-inflammatory cytokines in *plasmodium*-infected animals, this study found that no significant changes at the tissue level have been observed. Curcumin can only suppress hepatic necrosis in the experimental animals.

This research has some limitations in that the researchers only focus on qualitative identification of the morphological changes in the liver without taking into consideration the other laboratory aspects. Consequently, the researchers are unable to correlate the therapeutic effects of *Areca nuts* on the liver and the systemic conditions that occur in *P. berghei* infection. Changes in the hepatic function (such as AST and ALT levels) during *P. berghei* infection have not been identified either. However, this study has been able to demonstrate the potential of *Areca nut* extract as malaria therapy at the morphological level. This scientific evidence can become the basis of malaria research development in conjunction with the use of *Areca nut* extract. Further research is required to identify the range of therapeutic dose and toxic dose of *Areca nut* extract as herbal therapy for malaria in experimental animals.

CONCLUSION

The administration of *Areca nut* ethanol extract alone in a short term can provide better histopathological features than those of curcumin therapy alone or combination therapy.

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REFERENCES

1. Scaccabarozzi D, Deroost K, Corbett Y, Lays N, Corsetto P, Salè FO, et al. Differential Induction Of Malaria Liver Pathology In Mice Infected With Plasmodium chabaudi AS or Plasmodium berghei NK65. Malar J. 2018;17(1):1–9.
2. Autino B, Corbett Y, Castelli F, Taramelli D. Pathogenesis of Malaria in Tissues and Blood. Mediterr J Hematol Infect Dis. 2012;4(1).
3. Guha M, Kumar S, Choubey V, Maity P, Bandyopadhyay U, Guha M, et al. Apoptosis In Liver During Malaria: Role of Oxidative Stress and Implication of Mitochondrial Pathway. FASEB J. 2006;20(8):1224–6.
4. Deroost K, Lays N, Pham TT, Baci D, Van Den Eynde K, Komuta M, et al. Hemozoin Induces Hepatic Inflammation in Mice and Is Differentially Associated with Liver Pathology Depending on the Plasmodium Strain. PLoS One. 2014;9(11):1–23.
5. jiang, jing-hua, jung, suk-yul, kim, youn chul, shin, sae ron, yu, seung taek, park H. Antimalarial Effects of Areca catechu L. J Physiol Pathol Korean Med. 2009;23(2):494–8.
6. Sarpangala KB, Sarpangala M, Devasya A. Antimicrobial Properties of Areca Nut, Areca Catechu, L: A Review. Int J Res Ayurveda Pharm. 2017;8(3):8–12.
7. Cahyanto HA. Aktivitas Antioksidan Ekstrak Etanol Biji Pinang (Areca catechu, L). Maj BIAM. 2018;14(2):70.
8. Prasetyo Y, Suyatmi, Hanim D. Pengaruh Pemberian Ekstrak Kunyit Kuning (Curcuma longa) dalam Mencegah Kerusakan Hepar Mencit yang Diinduksi Alkohol. Biofarmasi J. 2012;10(1):28–33.

9. Ali AH, Sudi S, Basir R, Embi N, Sidek HM. The Antimalarial Effect of Curcumin Is Mediated by the Inhibition of Glycogen Synthase Kinase-3β. J Med Food. 2017;20(2):152–61.
10. Sari MS, Ridwan A. Efektivitas Ekstrak Etanol Biji Pinang Terhadap Densitas GLUT4 pada Sel-Sel Otot Rangka Mencit yang Terinduksi Hiperglikemia. J Sumberd Hayati. 2016;2(2):52–8.
11. Siegmund, Britta; Lear-Kaul, Kelly C.; Faggioni, Raffaella; Fantuzzi G. Leptin deficiency, not obesity, protects mice from Con A-induced hepatitis. eur J immunol. 2002;32:552–60.
12. Fitri F, Sekolah H, Ilmu T, Samarinda K. Uji Aktivitas Ekstrak Etanol Biji Pinang (Areca catechu L.) Terhadap Penyembuhan Luka Bakar Pada Kulit Punggung Mencit Putih Jantan (Mus musculus). Jurnal Ilmiah Manuntung, 2(2), 154–160.
13. Bone M, Fridayanti A, Rijai L. Potensi Antidiare Ekstrak Etanol Biji Pinang (Areca catechu L.) Terhadap Mencit Putih. 2015;178–90.
14. Chang LY, Wan HC, Lai YL, Kuo YF, Liu TY, Chen YT, et al. Areca nut Extracts Increased Expression of Inflammatory Cytokines, Tumor Necrosis Factor, Interleukin-1, Interleukin-6 And Interleukin-8, in Peripheral Blood Mononuclear Cells. J Periodontal Res. 2009;44(2):175–83.
15. Lai Y-L, Wu C-Y, Lee Y-Y, Chang H-W, Liu T-Y, Hung S-L. Stimulatory Effects of Areca Nut Extracts on Prostaglandin E 2 Production by Human Polymorphonuclear Leukocytes . J Periodontol. 2010;81(5):758–66.
16. Sari LM, Suyatna D, Utami S, Chairul C, Subita GP, Whulandhary YS, et al. Acute Oral Toxicity Study of Areca catechu linn. Aqueous Extract in Sprague-Dawley Rats. Asian J Pharm Clin Res. 2014;7(5):20–2.
17. Arjana AAG, Sukada IM, Suratma NA. Betel nut (Areca catechu L.) Extract Toxicity On The Kidney And Liver Of White Rats (Rattus norvegicus). Bali Med J. 2019;8(2):535.
18. Percário S, Moreira DR, Gomes BAQ, Ferreira MES, Gonçalves ACM, Laurindo PSOC, et al. Oxidative stress in Malaria. Int J Mol Sci. 2012;13(12):16346–72.
19. Husna M, Prasetyo BH. Aspek Biomolekuler dan Update Terapi Malaria Serebral. J MNJ. 2016;(02)(02):79–88.
20. Budhi K, Soemantri A, Aminullah A, Suhartono S. Kadar Antioksidan Rendah Meningkatkan Risiko Hemolisis pada Sepsis Neonatus. J Kedokt Brawijaya. 2011;26(3):180–4.
21. Ewbank AC, Strefezzi RDF, Sacristán C, Miyaji Kolesnikovas CK, Martins A, Mayorga LFSP, et al. Comparative Morphometric Evaluation Of Hepatic Hemosiderosis In Wild Magellanic Penguins (Spheniscus magellanicus) infected With Different Plasmodium spp. Aubgenera. Rev Bras Parasitol Vet. 2019;28(1):68–79.
22. Dewi ZS, Zam ZZ, Rakhman KA, Dewi ZS, Zam ZZ, Rakhman KA. Maserasi dan Uji Aktivitas Ic 50 Antioksidan Buah Pinang (Areca catechu) Secara SpektrofotometrI UV-VIS. Jurnal Saintifik.2017;1:6.