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The Effect of Combination of Sambiloto and Spirulina Extract on Cyclooxygenase-2 (COX-2) Protein Expression in Medial Colon of Mice Infected with Plasmodium *Berghei ANKA*

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Abstract

Currently, malaria is still a world health problem, including Indonesia. The trend of increasing cases of resistance to first-line antimalarial drugs adds to the list of problems with this disease. Sambiloto (*Andrographis paniculata*) and spirulina (*Arthrospira platensis*) are two herbal plants that are widely available in Indonesia. These plants have anti-inflammatory activity and have the potential as a new alternative to anti-malarial drugs. This study was conducted to determine the effect of the combination of both plants on the expression of Cyclooxygenase-2 (COX-2) protein, as a marker of inflammation, in the medial colon of malaria model mice. Thirty male *Swiss Webster* mice infected with *Plasmodium berghei* ANKA were divided into five groups: Sambiloto extract (AP), a combination of sambiloto and spirulina extract (AP + ES), a combination of sambiloto extract and spirulina powder (AP + PS), positive control (DHP), and negative control (CMC). A total of five colon's histological images from each sample were taken by microscope (400x) and analyzed using Image J to obtain the percentage of the H-Score index value of COX-2 expression. Significant results were found between the DHP group with AP, AP+ES, and AP+PS ($p<0.05$). The mean value of COX-2 expression in the DHP, AP, AP+ES, and AP+PS groups were 226.67; 201.89; 203.22; and 204.9, respectively. It can be concluded that the administration of sambiloto and spirulina, either in a single form (AP) or in combination (AP+ES and AP+PS) can reduce COX-2 expression in the medial colon of mice infected with *Plasmodium berghei* ANKA.

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Introduction

Malaria is a life-threatening disease and is often found in many tropical and subtropical areas, including Indonesia [1]. In 2015, eastern Indonesia was still the region with the highest annual parasite incidence (API) rate because the percentage and number of endemic districts/cities were mostly concentrated in the eastern region [2,3]. Every year, the number of cases of resistance to antimalarial drugs is increasing [3]. All geographic areas have this resistance with the most severe areas being in Southeast Asia [4]. As resistance to the first-line antimalarial drug, artemisinin, is growing, new alternative treatments need to be developed [5,6].

Indonesian society is very close to traditional medicine derived from plants, for example, sambiloto and spirulina [3]. Both of these plants have potential as therapeutic options in treating malaria. Sambiloto (*Andrographis paniculata L.*) is known to have antiplasmodial and anti-inflammatory activity. The c-phycocyanin as one of the ingredients of spirulina (*Arthrospira platensis*) can give protection against oxidative damage to DNA plasmid proteins and erythrocyte membranes, and also can inhibit the formation of proinflammatory cytokines [3,7]. Spirulina is used in two dosage forms, namely, extract and powder. Both of these dosage forms were used in this study to compare their effectiveness when combined with sambiloto.

Inflammatory response due to systemic infection of *Plasmodium sp.* can occur in various organs of the body, one of which is the colon [3,8]. In the previous study, the results showed that giving a combination sambiloto and spirulina extract significantly reduced the number of inflammatory foci in the colonic tissue of mice infected with *Plasmodium berghei ANKA* [3]. The reduction in the number of inflammatory foci certainly indicates an improvement in the malaria-infected animal. This also further supports the combination of natural ingredients of sambiloto and spirulina as a new alternative to malaria curative therapy which has a positive effect on disease improvement, which is decreasing inflammatory processes in the colon.

Based on the above phenomena, the researchers are interested in further researching how the effect of the combination of sambiloto and spirulina extract on one of the prognostic indicators of malaria, the expression of proinflammatory Cytokines Cyclooxygenase-2 (COX-2) in the medial colon of malaria model mice. The observation is specifically focused on the expression of COX-2 because this protein will increase when inflammation occurs so that it can be used as a marker or indicator of inflammatory events. Therefore, this study aims to prove the effect of giving a combination of sambiloto and spirulina extract and to test the effectiveness of spirulina in the form of extract and powder when combined with sambiloto extract on the expression of COX-2 protein in the colon media of mice infected with *Plasmodium berghei ANKA*.

Methods

Subjects

This study was an experimental study conducted in the laboratory to determine the effect of giving a combination of sambiloto and spirulina extract on the expression of COX-2 protein in the colon media of mice infected with *Plasmodium berghei ANKA*. The research subjects were male Swiss Webster mice from the Animal Laboratory of the Research and Development Center of the Ministry of Health in Indonesia who had been given treatment. The research sample was thirty male Swiss Webster mice, aged 8-10 weeks with a weight of 24-28 grams, infected with Plasmodium berghei ANKA, and given worm medicine.

Materials

The materials used include materials for extract characterization including distilled water, 95% ethanol (Merck), hydrochloric acid (Merck), methanol (Merck), concentrated sulfuric acid (Merck), acetic acid (Merck), toluene (Merck), ethyl acetate (Merck), chloroform (Merck), vanillin sulfate (Merck), hexane, indigo sulfuric acid (Merck), ammonium sulfate (Merck), andrographolide (Sigma), and the quantifier quercetin (Sigma). Silazine, ketamine, 70% ethanol, citrate buffer, and formalin are also used. Other ingredients used include ethanol extract 80% spirulina, ethanol extract 70% sambiloto, DHP tablets (dihydroartemisinin-piperaquine), Na-CMC, immersion oil, PBS, EDTA, and primary antibodies used include polyclonal antibody COX-2. Sambiloto herbs were obtained from the Research Institute for Spices and Medicines, while the spirulina test material in the form of powder was the product of PT. Trans Food Spirulindo Jepara, Central Java.

Research groups

The sample of this study was divided into five groups, with three as a test group and two as a control group, consisting of the Sambiloto extract (AP), the sambiloto extract and the spirulina extract (AP+ES), the sambiloto extract and the spirulina powder (AP+PS), negative Control Carboxymethyl Cellulose (CMC), and positive control Dihydroartemisinin-Piperaquine (DHP) groups.

Preparation of 70% ethanol extract of sambiloto

Sambiloto simplicial obtained was dried, then powdered and sieved using a 40 mesh sieve. The powder was then weighed as much as 250 g, moistened using 70% ethanol solvent and allowed to stand for 18 hours in a closed glass container. The macerate was filtered and the residue was macerated again using 70% ethanol. This maceration is carried out in three stages. The final filtrate obtained is then collected and thickened using a rotary evaporator, the viscous extract is evaporated in a porcelain dish which has been tared, then evaporated over a water bath with a temperature of ± 40°C so that the ethanol evaporates and finally the dry extract is obtained.

Preparation of 80% ethanol extract of spirulina

100 g of spirulina powder is macerated using 500 ml of 80% ethanol, then accompanied by stirring for about 20 minutes at 60°C. The filtrate is filtered and the residue is then remacerated twice. This method of extraction produces the most total fatty acids. Extraction of spirulina using ethanol produced the components with the highest antioxidant activity, including phenolics and flavonoids.

Calculation of the dosage

DHP dose for positive control was 195 mg/kg BW dissolved in 0.5% Na-CMC from day 1 to day 4. The dose of 70% sambiloto ethanol extract is 200 mg/kg BW which has been in accordance with the sub chronic safe dose and is given from three days before parasite induction (D-3) until the 28th day after induction. The spi-

rulina powder dosage is 130 mg/kg BW based on the sub-chronic toxicity test of mice which is safe to use for 28 days of administration. The spirulina extract dosage given refers to the spirulina powder dosage multiplied by the percent yield of ethanol extract, which is 20%, so the dosage is 26 mg/kg BW.

COX-2 immunohistochemical staining

Medial colonic tissue that had been made in paraffin blocks were cut 4 µm thick and placed on a poly-L-lysine-coated glass object for further Immunohistochemical (IHC) staining. IHC procedure is carried out through deparaffinization, rehydration, antigen retrieval, and blocking processes. After that, the preparations were incubated at room temperature with anti-COX-2 antibodies in Phosphate Buffer Solution (PBS) for 2 hours then visualized using diaminobenzidine (DAB) for 20-30 seconds. The preparation was then immersed in a Lilie Mayer Hematoxylin solution for approximately 1-2 minutes which acts as a counterstain. Then the tissue preparation is immersed in lithium carbonate for 1 minute. After that, dehydration was carried out using ethanol and clearing using xylol. Finally, it is closed with aqueous mounting media. The stained preparations were examined for histopathological change.

Quantification of cox-2 protein expression

All tissue preparations were observed using a 400X magnification microscope which was then documented using a build-in camera attached to the microscope. Observations in one sample were taken as many as five fields of view randomly. The results will be interpreted in the form of a percentage of strong positive results (dark brown +++), positive (brown ++), weak positive (light brown +), and negative (blue) using the ImageJ® application with the IHC profiler program and declared in the H-Score. The results of the H-Score based on the following formula: (%high positive × 4) + (%positive × 3) + (%low positive × 2) + (%negative).

Data analysis

Statistical analysis in this study using the SPSS 26.0 with a 95% confidence interval. Descriptive statistics are presented in mean ± standard deviation. Data were Analyzed Using Parametric Analysis of Variance (ANOVA) test then followerd by Bonferroni's Post Hoc test to find out which groups had differences. P value less than 0.05 (P<0.05) are considered statistically significant.

Results

Quantitative characteristics of samples

The results of the Shapiro-Wilk normality test on the COX-2 expression level in 29 samples showed that the data were normally distributed (p>0.05). Figure 1 shows the distribution of the data as a whole. Because the data are normally distributed, descriptive statistics are presented in mean ± standard deviation as a representation of the overall data. Further research descriptive data are shown in Table 1.

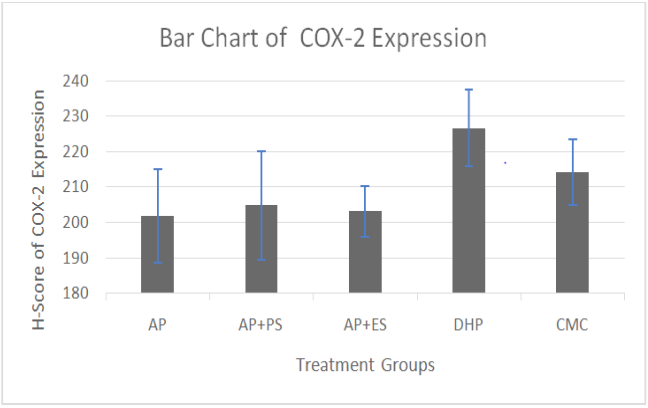

Figure 1: Bar chart of mean COX-2 expression for each treatment group with error bars as standard deviation.

Table 1: H-Score data of COX-2 expression for each treatment groups.

	Mean ± SD	p-value
Sambiloto Extract (AP)	201.89 ± 13.1	0.005
Sambiloto Extract and Spirulina Ekstract (AP+ES)	203.22 ± 7.18	
Sambiloto Extract and SpirulinaPowder(AP+PS)	204.9 ± 15.27	
Dihydroartemisinin-piperaquine (DHP)	226.67 ± 10.81	
Carboxymethyl Cellulose (CMC)	214.19± 9.2	

Correlation test between independent variables (AP, AP + ES, AP + PS, DHP, and CMC) with the dependent variable (COX-2 expression) based on the Bonferroni's Post Hoc test showed significant results (p<0.05).

Table 2: The Bonferroni's test p-value of COX-2 expression between groups.

	AP	AP+ES	AP+PS	DHP	CMC
AP	1	-	-	-	-
AP+ES	1	1	-	-	-
AP+PS	1	1	1	-	-
DHP	0,015	0,016	0,03	1	-
CMC	0,875	1	1	0,7	1

Based on table 2, some significant results were found. The difference in COX-2 expression was found to be significant between the DHP and AP, DHP with AP + ES, and DHP with AP + PS groups (p<0.05). The DHP treatment group did not show significant results with the CMC group (p>0.05). The CMC treatment group also did not show a significant difference from the AP group (p> 0.05).

Qualitative characteristics of sample

Qualitatively, the level of COX-2 expression in the medial colonic tissue of mice is grouped into four groups, namely high positive (+++), positive (++), low positive (+), and negative (-). This qualitative value is obtained through automatic analysis of ImageJ® software by calculating the percentage of cells with high

positive, positive, low positive and negative values in a field of view. These displayed qualitative characteristics do not necessarily replace quantitative assessments of COX-2 expression levels and are only a supplement to demonstrate that ImageJ® contributes to qualitative assessments.

Some of the observations of preparations with a microscope (400x) are presented in Figure 2. Comparison of the qualitative picture between the low positive and positive results based on ImageJ® can be seen in Figure 2. It is worth recalling that ImageJ® assesses the level of COX-2 expression based on the overall color gradient of the image which is not necessarily can be done by human. Therefore, the qualitative assessment of the expression level manually on each epithelial cells and then summed cannot be compared with the results of the ImageJ® algorithm assessment. A comparison of the immunohistochemical features in the five test groups is presented in Figure 3.

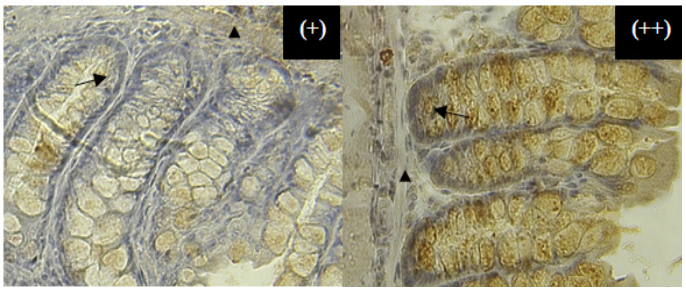

Figure 2: Comparison of the qualitative expression of COX-2 preparations with 400x magnification based on ImageJ: low positives are generally bluish brown (left), while positives are generally dark brown with no bluish spots (right). The arrows (→) refer to epithelial cells, while the triangles (▲) refer to the lamina propria**.**

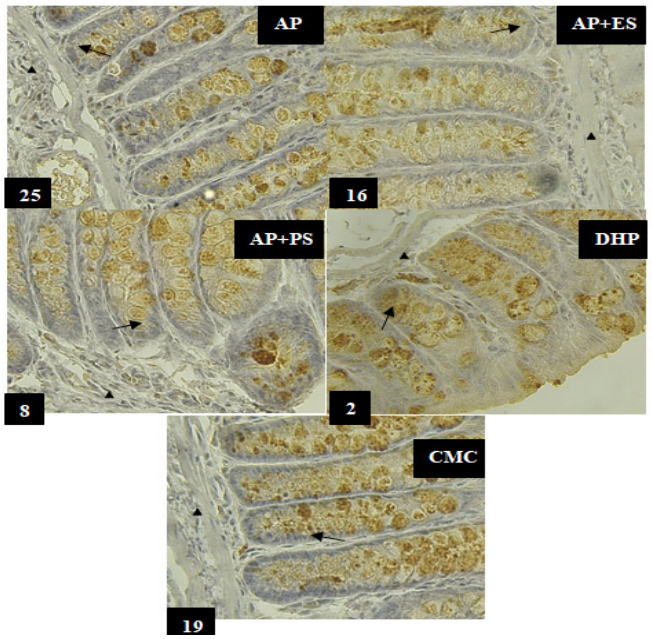

Figure 2: Photo comparison of medial colonic tissue preparations in the five test groups. The label in the upper-right corner indicates the test group, while in the lower-left corner the number of the preparation. The arrows (→) refer to epithelial cells, while the triangles (▲) refer to the lamina propria.

Discussion

Dihydroartemisinin-piperaquine (DHP)

Dihydroartemisinin-piperaquine (DHP) is one of five artemisinin-based combination therapy (ACT) recommended by WHO for treating malaria at this time [9]. In Indonesia, since 2009, the provision of ACT in the form of DHP has become the main recommendation for therapy uncomplicated malaria because it can increase effectiveness and prevent resistance, plus primaquine which acts as a gametocidal and hypnozoidal [10,11]. This combination contains artemisinin components (such as Dihydroartemisinin) which work very quickly to eliminate malaria parasites from the blood of an infected person and drugs with a long half-life time (such as piperaquine) which can clear the traces of parasites in the blood and can prevent new malaria infections for several weeks [9,12]. Other studies have also shown that the clinical and parasitological response of DHP in uncomplicated malaria patients is very good on day 3 [13]. Therefore, DHP was used as a positive control in this study and administered for four days after the induction of *Plasmodium berghei ANKA*.

Sambiloto extract (AP)

Provision of sambiloto extract (AP) from three days before the induction of *P. berghei* (D-3) to the 28th day after induction (D28) gave significant results ($p = 0.015$) on COX-2 expression in the medial colon of mice compared with the DHP positive control group. *Andrographis paniculata* (sambiloto) has several ingredients in it. The active compounds of sambiloto are extracted with ethanol or methanol in all parts of the plant and there are more than 20 terpenoid compounds, 10 flavonoids, steroids, and saponins [7,14]. Andrographolide ($C_{20}H_{30}O_5$) is the main active component of the diterpenoid group [7,14-16]. The other main terpenoid compounds are deoxyandrographolide, neoandrographolide, isoandrographolide, and 14-deoxy-11,12-didehydroandrographide [14-16].

The main active compound of andrographolide has various pharmacological properties, one of which is an anti-inflammatory effect [14-16]. Inflammation involves activation of macrophages and T lymphocytes, as well as the release of proinflammatory mediators, such as IL-1, IL-6, IFN-γ, TNF-α, Nitric Oxide (NO), and cell adhesion molecules which then amplify the inflammatory process [14]. Overproduction of NO and PGE2 due to the expression of inducible is forms of NO synthase (iNOS) and COX-2 plays an important role in the inflammatory process [17]. A study showed that administration of the methanol extract of sambiloto intraperitoneally for five consecutive days succeeded in inhibiting 65% of NO production by peritoneal macrophages [14,16,17]. In addition, sambiloto is also able to inhibit the production of oxygen radicals on neutrophils, inhibits macrophage migration, NFκB activity, and TNF-α production and IL-12 [14,16,18]. The anti-inflammatory activity of andrographolide is thought to result from interference with protein kinase C dependent pathways, regulation of extracellular signal kinase 1/2 (ERK1/2), or PI3K / Akt signaling pathways. Neoandrographolide compounds can also suppress NO production and inhibit the production of TNF-α and PGE2 [14,16,19,20]. The andrograpanin component which is a neoandrographolide hydrolyzate is also able to reduce the production of NO, TNF-α, and IL-6. The ethanol extract of sambiloto has also been shown to suppress the production of IL-6, TNF-α, macrophage inflammatory protein-2 (MIP-2), PGE2, and NO [14].

The main activity of andrographolide is to inhibit the binding of NFκB to DNA so that it will reduce the expression of proinflammatory proteins, such as COX-2 and nitric oxide synthase (NOS) [16,18,20]. NFκB has an important role in the pathogenesis of inflammation so that many drugs are designed to focus on inhibiting the activation of NFκB [17]. An important marker in explaining the mechanism of action of andrographolide is the fact that andrographolide suppresses cysteine 62 from p50 (the main subunit of transcription factor NFκB) so that it can block its binding to the promoter of the target gene [21]. One of the target genes whose promoter contains the NFκB element is the COX-2 gene. The COX-2 gene is located on chromosome 1 and the promoter of the COX-2 gene indicates that there are NFκB response elements as well as other cytokine-dependent response elements (such as IL-6) [22].

Combination of sambiloto and spirulina (AP+ES and AP+PS)

Giving a combination of sambiloto and spirulina extract, either in the form of extract (AP+ES) or powder (AP+PS) given from three days before induction of *P. berghei* (D-3) until the 28th day after induction (D28) gives significant results. The AP+ES treatment group had significant results ($p = 0.016$) against the DHP group, as well as the AP+PS treatment group ($p = 0.03$). The addition of spirulina both in the form of extract and powder also gave an anti-inflammatory effect by reducing the level of COX-2 expression in the medial colon of *P. berghei* infected mice, although the addition of spirulina was not significantly different from the treatment group which only received sambiloto extract ($p = 0.015$).

Arthrospira platensis (spirulina) has a high content of c-phycocyanin compounds. Several studies demonstrate the anti-inflammatory function of this compound. The c-phycocyanin component is said to have inhibitory activity against COX-2 which is highly expressed in times of inflammation. The inhibitory activity of COX-2 is known through the attachment mechanism of c-phycocyanin with VEGF1 which plays a role in the angiogenesis process [23]. C-phycocyanin significantly inhibits COX-2 with an IC50 value of 80 nM [24]. The anti-inflammatory activity of c-phycocyanin may be due to its selectivity in inhibiting COX-2, but studies have also suspected this is due to the involvement of spirulina's ability to eliminate free radicals and inhibit lipid peroxidation [7,24].

Many studies have demonstrated the anti-inflammatory effects of spirulina. Molecular studies have shown that c-phycocyanin can down regulate the level of mRNA expression of the COX-2 gene in mice [25,26]. Semiquantitative RT-PCR analysis of the mRNA levels of inflammation-related genes has shown a significant suppression of inflammatory factors, such as TNF- α and MIP-3a when given a polysaccharide extract from spirulina [27]. Other studies have also shown that c-phycocyanin in spirulina can reduce levels of PGE2 [28,29]. Research in diabetic mice also shows the anti-inflammatory effect of spirulina, namely by reducing levels of the inflammatory mediator TNF- α and IL-6 [29-31]. Research on the inhibitory effect of spirulina on inflammatory markers, such as Myeloperoxidase (MPO) and also the proinflammatory cytokine IL-1β has also been widely known [29,32]. Similar effects to sambiloto were also found in spirulina. The administration of spirulina to experimental animals significantly reduced the activation of NFκB [33,34]. The other anti-inflammatory effects of spirulina as well It has been known to inhibit the release of histamine by mast cells [35]. Apart from phycocyanin, other ingredients in spirulina, namely β-carotene also have the ability to reduce inflammatory processes such as phycocyanin [36].

The anti-inflammatory activity in spirulina has been proven by many studies in line with the results of this study. This. The level of COX-2 expression in the treatment group that was given a combination of sambiloto and spirulina extract, both in extract and powder form, decreased significantly when compared to the DHP control group. Although in previous studies, it was found that there were significant results in the number of inflammatory foci between the groups that were added with spirulina administration compared to the group that only received sambiloto extract, this is not the case with the results obtained in this study. This study shows that at the level of inflammatory markers, namely COX-2, both the group that was given only bitter extract (AP) and the group combined with spirulina (AP + ES and AP + PS) had similar activity in reducing the level of COX-2 expression.

Limitations of the study

The limitations of this study are that it is not certain about the pharmacokinetics and pharmacological effects of each spirulina extract and powder dosage form, and it does not involve a group of experimental animals that are not infected with malaria (normal control group).

Conclusion

Based on the research results, it can be concluded that the administration of sambiloto extract (AP), a combination of sambiloto and spirulina extract (AP+ES), and a combination of sambiloto extract and spirulina powder (AP+PS) were proven to reduce the expression of COX-2 protein in the colonic media of mice infected with *Plasmodium berghei ANKA* compared with a DHP positive control group. However, there was no significant difference in effectiveness between the administration of the combination form of sambiloto extract and spirulina extract (AP+ES) with the combination of sambiloto extract and spirulina powder (AP+PS).

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