

The Effect of Sambiloto and Spirulina Combination on Mucin-1 Protein Expression in Medial Colon of *Plasmodium berghei* ANKA Infected Mice

Kusmardi Kusmardi^{1-4,*}, Baiqi Nur Hairi⁵, Nadar Sukri Lubis⁶, Tri Wahyuni Lestari⁷, Putri Reno Intan⁸

Kusmardi Kusmardi^{1-4,*}, Baiqi Nur Hairi⁵, Nadar Sukri Lubis⁶, Tri Wahyuni Lestari⁷, Putri Reno Intan⁸

¹Anatomical Pathology Department, Faculty of Medicine, Universitas Indonesia, Jakarta, INDONESIA.

²Drug Development Research Cluster, Indonesia Medical Educational and Research Institute, Jakarta, INDONESIA.

³Human Cancer Research Cluster, Indonesia Medical Educational and Research Institute, Jakarta, INDONESIA.

⁴Doctoral Program in Biomedical Sciences, Faculty of Medicine, Universitas Indonesia, Jakarta, INDONESIA.

⁵Undergraduate Program, Faculty of Medicine, Universitas Indonesia, Jakarta, INDONESIA.

⁶Parasitology Department, Faculty of Medicine, Universitas Indonesia, Jakarta, INDONESIA.

⁷National Institute of Health Research and Development, Ministry of Health of Indonesia, Jakarta, INDONESIA.

⁸Centre for Research and Development of Biomedical and Basic Health Technology, National Institute of Health Research and Development, Ministry of Health, Jakarta, INDONESIA.

Correspondence

Kusmardi Kusmardi

Anatomical Pathology Department, Faculty of Medicine, Universitas Indonesia; Drug Development Research Cluster, Indonesia Medical Educational and Research Institute; Human Cancer Research Cluster, Indonesia Medical Educational and Research Institute, Jakarta, INDONESIA.

E-mail: kusmardi.ms@ui.ac.id

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ABSTRACT

Malaria still be health problem in the world, especially in Eastern Indonesia. Malaria's inflammation and metabolism defect can cause colonic damage, such as enhancement Muc-1 protein expression and goblet cells hyperplasia. Sambiloto and spirulina combination as antiinflammatory and antioxidative agent can prevent medial colon damage *Plasmodium berghei* ANKA infected mice. The aim of the study to show the effect of sambiloto and spirulina combination on Muc-1 protein activity in medial colon *Plasmodium berghei* ANKA infected mice. This study use preserve male Swiss Webster mice colonic tissue which has inoculated by *Plasmodium berghei* ANKA, whose treatment group include positive control (dehydroartemisin piperazine), negative control (carboxymethyl cellulose), AP (sambiloto), AP+ES (sambiloto+spirulina extract), and AP+PS (sambiloto+spirulina powder) and terminated after 28 days of treatment. Colonic tissue was stained with immunohistochemistry and observed using light microscope (400x) in five different field and was analyzed with ImageJ® software, and statistical analysis was done with SPSS 20.0. According to One Way ANOVA and Duncan posthoc test, only AP+PS(120,98 ±3,37), which significantly difference between negative control, AP, and AP+ES group. Meanwhile, between DHP, AP+PS group not significantly difference. Sambiloto extract and spirulina powder combination can reduce Muc-1 protein expression in medial colon *Plasmodium berghei* ANKA infected mice.

Key words: Medial colon, Muc-1, *Plasmodium berghei* ANKA, Sambiloto, Spirulina.

INTRODUCTION

Malaria is a health problem which has been concered in Indonesia and the world. In Indonesia, according to malaria's cumulative incidence/ Annual Parasite Incidence (API) in the 2015, Indonesia have 0.85 of 1000 people who infected by malaria, with in the Eastern of Indonesia has 31.29-31.93 of 1000 people who infected by malaria.¹ Meanwhile, malaria eradication has been planned in target points number 3 related to the provision of a healthy life and health promotion in the Sustainable Development Goals (SDGs) by the United Nations²

Malaria can indirectly attack various organs, one of which is the intestine. Malaria-infected erythrocytes block the intestinal villi resulting in digestive tract destruction and metabolic defects. The inflammatory process cause increased oxidative stress, goblet cells hyperplasia, increased expression of Mucin (Muc) -1, Muc-4,³ and Muc-1⁴, aswell as a shortening of vili.⁵

As an innate immunity, mucus consists of mucin network. Muc-1 is kind of mucin whose functions to prevent infection and colonization as well as pathogen-induced inflammatory modulation processes. In the gastrointestinal tract, Muc-1 prevents intestinal inflammation and infection of *Campylobacter jejuni*⁴, prevents colonization and adhesion of *Helicobacter pylori*, while preventing the effects of these pathogens to gastric mucose.^{4,6} Although no studies have looked for the mucin protein expression on *Plasmodium berghei* ANKA (PbA), *Plasmodium berghei* ANKA infected mice results in thickening of the mucus in the colon.⁵

Meantime, Muc-1 also has a role to be markers of interstitial lung inflammation, such as COPD, asthma, and pneuomonia, and the protein expression is found in the inflammatory bowel disease.⁴

The latest therapy for malaria is a artemisinin combination therapy (ACT). ACT prevents recurrent parasitemia before day 28, longer the prophylactic effect, reducing symptoms faster than monotherapy and chloroquine.⁷⁻⁹ However, even its in common, ACT treatment can cause side effects, such as nausea, vomiting, stomach pain, and even black urine.⁷

As an alternative to malaria treatment, herbal plants have been one of the options. One of the potential plants is sambiloto (*Andrographis paniculata*). Sambiloto contains andrographolide which is antimicrobial, anti-inflammatory, and anti-allergy. As an antimalarial, this plant has an efficacy similar to chloroquine and artemisin and has a role in inhibition of the malaria ring phase and reduces parasitemia.¹⁰ Sambiloto also acts as a mucus barrier by preventing mucus reduction in gastric ulcers.¹¹ Meanwhile, other herbs act as antioxidants in infections, such as *Spirulina platensis*, whose potential role in protecting against oxidative stress^{12,13}

Sambiloto and spirulina combination therapy has a potential synergistic role in malaria therapy. The combination of sambiloto extract and spirulina powder in mice infected with *Plasmodium berghei* ANKA can inhibit parasitemia, increase the number of erythrocytes and significant hemoglobin levels compared to sambiloto monotherapy alone.¹² While in the colon, the combination of these two herbs was not significant in increasing goblet cells, but reduced

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inflammation and angiogenesis processes compared to sambiloto monotherapy.¹⁴ When compared with the combination of sambiloto extract and ethanol extract of spirulina, the combination of sambiloto and spirulina powder significantly reduced parasitemia¹², but had the same effectiveness in reducing inflammatory focus and angiogenesis in the colon of PbA-infected mice.¹⁴

Therefore, the researchers are interested in examining the relationship between sambiloto and spirulina, in powder and extract, combination to the activity of Muc-1 mice infected with *Plasmodium berghei* ANKA. Researchers wanted to see the effectiveness of the combination of these two herbs compared to monotherapy and DHP, and to compare the effectiveness of sambiloto extract and spirulina powder with sambiloto extract and spirulina extract in influencing the activity of Muc-1 mice infected with *Plasmodium berghei* ANKA.

METHODS

Research sample

This study is an experimental study that utilizes preserve biological material in the form of colon tissue blocks in the media of male mice Swiss Webster from the Animal Laboratory of the Health Research and Development Agency, Ministry of Health, Jakarta, aged 8-10 weeks with a weight of 24- 28 grams have been infected with PbA, were given deworming medicine, and have been given treatment from previous published research.¹⁵ Immunohistochemical staining using Anti-Muc-1 which has been made in the preparation. Samples were excluded if there was no evidence of adequate colonic media on each of the preparations used for observation in the five visual fields.

There were 5 groups in this study that had PbA induction on day 0, where the number of samples for each group was 5. The group included a negative control group (CMC) using 0.5% sodium carboxymethyl cellulose from day 1 to 28, the control group positive (DHP) by administering DHP 195 mg / kgBW with 0.5% Na-CMC solvent given from days 1-4.¹⁵ The first herbs group was sambiloto monotherapy (AP) using 70% ethanol extract of sambiloto at a dose of 200 mg / kgBW according to the subchronic toxicity dose.^{15,16} The second herbs group is a combination group of 70% sambiloto ethanol extract 200 mg / kgBW and 130 mg / kgBW spirulina powder in 0.5% Na-CMC, which is a subchronic safe dose.^{15,17} The last herbs group was a combination group of 70% sambiloto ethanol extract 200 mg / kgBW and 80% ethanol extract spirulina 26 mg / kgBW in 0.5% Na-CMC.^{15,17}

Tools and materials

In this study, tools such as light microscopy, slide glasses, cover glasses, histopathology tools, coated object glasses of poly-L-lysine-, slide warmer, camera, and laptop were used. The materials were ethanol extract 70% sambiloto, ethanol extract 80% spirulina, DHP tablets, Na-CMC, immersion oil, Lillie Mayer hematoxylin, lithium carbonate, aqueous mounting media, phosphate buffer saline (PBS), xylol, solution endogenous peroxidase 0.5%, alcohol 95 and 80%, sniper blocking agent, TrekAvidin reagent, and immersion oil. Antibodies used in immunohistochemical processes include primary antibodies to anti-muc-1. Meanwhile, secondary antibodies Universal Link.

Preparation and immunohistochemistry

After the mice were terminated by initiating anesthesia using ketamine (75-100 mg / kg) ip and xylazine with a syringe whose needle measuring 24-26 GX 13-25 cm until terminated by neck dislocation technique, the colonic tissue of the mice was taken. This tissue is washed using water, then fixed with 10% formalin solution. Then, the sample was inserted into the paraffin medium, cross-cut with a microtome (4 µm thickness) for immunohistochemical staining.

The preparations to be histochemical stained were placed on a coated glass poly-L-lysine then heated on a warmer slide at 56.5-60 °C for 60 minutes and then deparaffinized with xylol 3 times. Then do rehydration with alcohol concentrations gradually, starting from 95% then 80% with a duration of 5 minutes. The preparations were washed with running water for 5 minutes, then immersed in an endogenous solution of 0.5% peroxidase for 30 minutes and rinsed again with running water for 5 minutes.

The next process is in the form of retrieval antigen for each finger in the citrate buffer pH 6 in the microwave for 10 minutes. After that, the pieces were left to stand for 30 minutes at room temperature, then washed for 10 minutes in solution phosphate buffer saline (PBS), then incubated with Universal Link secondary antibody for 15 minutes, and washed again for 10 minutes with PBS. Then, drop the TrekAvidin-HRP reagent and let it sit for 15 minutes at room temperature. The preparations were then washed with PBS 2 times with each washing time of 10 minutes. The preparation was then given a drop of diaminobenzidine chromogen (DAB) and let it steep for 20-30 seconds and immediately washed using running water. After that, for 1-2 minutes, the preparations were dipped in Lillie Mayer Hematoxylin solution as a counterstrain, then washed again with running water. The preparation was immersed in lithium carbonate for 1 minute, after which it was washed again under running water. Furthermore, the dehydration process with ethanol and cleaning with xylol then covered with aqueous mounting media.

Muc-1 protein expression assessment

Muc-1 protein expression was assessed based on the number of cell groups compared to the total cells in one field of view, which expressed as a percentage. The cell group consists of a strong positive / positive three (very brown), medium positive / two positive (brown), a weak positive / positive one (slightly brown), and negative (blue). Samples were observed with a 400 X light microscope, then documented with a camera. The observation process was carried out by single blind observer. Pictures were taken at 5 fields of view for each sample randomly. Assessment of Muc-1 protein expression interprets by the "IHC Profiler" plugin with ImageJ® software. The value of the Muc-1 protein expression level is based on the mean Histopathology score (H score) from 5 fields of view for each sample with the formula:

$$H\ Score: (4 \times \%(+3)) + (3 \times \%(+2)) + (2 \times \%(+)) + (\%(-))$$

Data analysis

Data were analyzed using SPSS for Windows version 20.0 with 95% confidence intervals with bivariate analysis comparing test treatment as independent variables and Muc-2 expression in the form of H score as the dependent variable. Data are presented as mean ± standard deviation. Samples were tested for normality using the Shapiro-Wilk test followed by Levene homogeneity test. Data were analyzed with analysis of variance (One Way ANOVA) followed by Duncan Posthoc test to compare the differences between therapy. P value <0.05 was assessed as a significant outcome on One Way ANOVA and Duncan's Posthoc test.

RESULTS AND DISCUSSION

In this study, there was a change in the number of samples from 30 to 29. This was because one of the preparations was excluded. From the AP + ES group, due to the cut process showing an image of the small intestine. The comparison of the images of the results of immunohistochemical staining in various test groups is presented in Figure 1.

Based on the results of the Shapiro-Wilk normality test, the data were normally distributed ($p > 0.05$) with a homogeneous data distribution using the Levene test ($p > 0.05$). The data distribution is shown in the bar chart in Figure 2, and the mean value is a data representation because the data is normally distributed. The mean value that describes

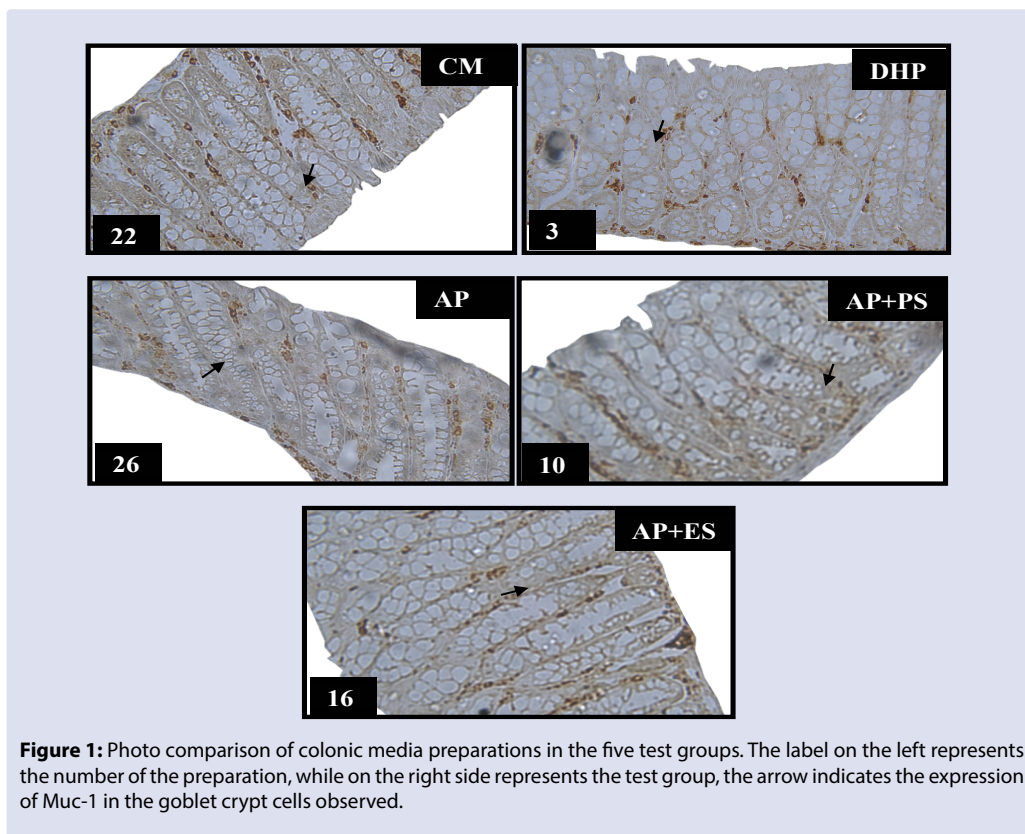


Figure 1: Photo comparison of colonic media preparations in the five test groups. The label on the left represents the number of the preparation, while on the right side represents the test group, the arrow indicates the expression of Muc-1 in the goblet crypt cells observed.

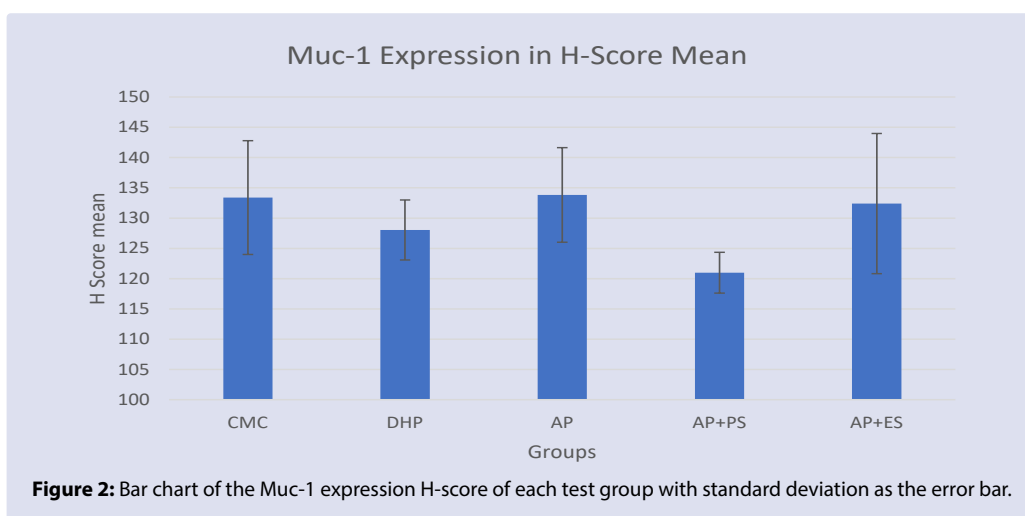


Figure 2: Bar chart of the Muc-1 expression H-score of each test group with standard deviation as the error bar.

the expression of Muc-1 is 129.64% with a standard deviation of 8.77%. The lowest value lies in the expression of Muc-1 protein in the AP + PS group.

According to the One Way ANOVA test, the hypothesis test of the relationship between giving bitter extract, spirulina extract, and spirulina powder had a significant result ($p < 0.05$). Meanwhile, based on Duncan's posthoc test presented in Table 2, the difference in mean Muc-1 expression had a significant result in the AP + PS group, which was compared with the sambiloto monotherapy (AP) group, negative control (CMC 0.5%), and combination of sambiloto with spirulina extract (AP + ES).

DHP therapy to Muc-1 Protein Expression in Mice Infected with *Plasmodium berghei* ANKA

Dihydroartemisin piperazine (DHP) is the first line drug for malaria management in Indonesia. Combinations of drugs increase malaria

eradication, efficacy, and prevent resistance.^{18,19} DHP is composed of a combination of artemisinin (dihydroartemisinin) and quinolone (piperazine) drugs. Dihydroartemisinin is a sesquiterpene lactone endoperoxide which acts as an oral schizonticidal agent by producing free radicals that cut the endoperoxide bridge catalyzed by food vacuole iron and inhibition of calcium ATPase disease prompts. Meanwhile, piperazine as bisquinone is commonly prescribed with DHP with the working mechanism of concentrating food vacuoles to prevent biocrystallization of Hb to hemozoin, which is toxic to parasites.^{18,20}

According to the results of the analysis, the test group had significance with the H-score of Muc-1 expression in mice infected with *Plasmodium berghei* ANKA. However, in the DHP group there was no difference between the various test and negative control groups. Therefore, it can be concluded that DHP administration can decrease Muc-1 expression but the effect is not different from the herbal group and negative control.

Table 1: Descriptive data of Muc-1 expression.

Group	N	Mean ± Standard Deviation	p-value
Negative control (CMC)	6	133,40 ± 9,40	
Positive control (DHP)	6	128,04 ± 4,94	
Sambiloto (AP)	6	133,84 ± 7,80	
Sambiloto and spirulina powder (AP+PS)	6	120,98 ± 3,37	<0,05
Sambiloto dan spirulina extract (AP+ES)	5	132,40 ± 11,59	
Total	29	129,64 ± 8,77	

Table 2: Duncan's posthoc test of Muc-1 protein expression between groups.

	CMC	DHP	AP	AP+PS	AP+ES
CMC	1	-	-	-	-
DHP	0,26	1	-	-	-
AP	0,26	0,26	1	-	-
AP+PS	<0,05	0,137	<0,05	1	-
AP+ES	0,26	0,26	0,26	<0,05	1

There are no studies that directly assess the effects of DHP on the Muc-1 protein. However, in Kusmardi et al, there was an insignificant increase in the number of goblet cells in PbA-infected mice that were given DHP. This increase in goblet cells is associated with mucus proteins as an inflammatory response and prevention of secondary bacterial infection.¹⁴ DHP is considered safe in long-term and intermittent administration. A dangerous side effect can be a prolonged heart rhythm QT interval. In addition, rashes and allergic reactions may occur.²⁰⁻²²

Combination of Sambiloto and Spirulina Powder or Spirulina Extract on Muc-1 Protein Expression in Mice Infected with *Plasmodium berghei* ANKA

Sambiloto and spirulina are herbal plants that have potential for malaria therapy. Sambiloto containing andrographolide can function as anti-inflammatory, antioxidant, immunostimulant, and anti-malarial.^{23,24} Meanwhile, spirulina is a blue-green algae containing c phycocyanin which is useful as an immunomodulator, antioxidant, and anti-inflammatory.^{13,25}

Based on the results, all herbs, both combination and sambiloto monotherapy, showed a decrease in Muc-1 protein expression. When compared with each group, the administration of sambiloto extract and spirulina powder had a significant reduction compared to the administration of sambiloto monotherapy, negative control, and the combination of sambiloto and spirulina extract. In addition, there was no significant difference between the decreased expression of Muc-1 protein in DHP with the combination of sambiloto extract and spirulina powder.

Mucin protein expression reduction in this study is different from previous studies that looked at the effect on the Muc-2 isomer. In Jiang et al study, administration of andrographolide (15 mg / kg and 45 mg / kg) prevented the reduction of muc-2 expression in DSS-induced colitis mice, restored colonic crypt, and prevented colonic shortening and ulceration.²⁶ According to Kusmardi et al., sambiloto extract and spirulina powder combination can increase goblet cells, but it is not statistically significant. The process of inflammation and hyperplasia of goblet cells and the addition of mucus glycoprotein molecules can prevent damage caused by inflammation and reduce the risk of bacterial adhesion.¹⁴

Sambiloto is indirectly synergistic with Muc-1 function in inflammation and immunity. Andrographolide reduces phosphorylation of ERK1 / 2 phosphorylation in murine T cells as well as lipopolysaccharide-induced TNF- α and GM-CSF inhibition. In addition, andrographolide

significantly inhibits NF- κ B attachment to DNA thereby decreasing cyclooxygenase-2 (COX-2) expression in endothelium, microglia, and kidney.²³ Andrographolide can also reduce proinflammatory cytokines, such as IL-1 β , IL-6, GM-CSF, and TNF- α .^{23,24} Sambiloto with leaf extract and andrographanin isolate has anti-inflammatory activity and inhibition of NO and proinflammatory cytokines ((TNF- α , IL-6, IL-12p70).²⁴ Muc 1 plays a role in suppressing NF- κ B activity in gastric epithelium which will inhibit inflammation against TNF- α associated with TLR and NOD-1 ligands. Muc-1 has the ability to prevent NF- κ B translocation and IL-8 production in the *Helicobacter pylori* therapy. The IL-8 inhibition process caused by Muc-1 modulation of NF- κ B with TNF and TLR / NOD receptors, on the IKK complex.²⁷ Increasing the amount of Muc-1 interferes with the penetration of the microbiota and the anti-inflammatory effect associated with TNF- α . Therefore, administration of sambiloto synergistically and indirectly can increase the expression of Muc-1 with effects on TNF- α and NF- κ B.

Spirulina in this study was combined with Sambiloto because of its antioxidant and anti-inflammatory effects. In the study of Kusmardi et al, the administration of sambiloto extract and spirulina powder was significantly decreased parasitemia compared to the combination of sambiloto and spirulina extract, seen from its efficacy compared to sambiloto monotherapy.¹² Both forms of this combination are effective in reducing the focus of inflammation and angiogenesis in the medial colon of PbA-infected mice.¹⁴ In this study, the experimental administration of sambiloto and spirulina extract did not differ from the herbal monotherapy, negative control, and positive control groups because the water-soluble antioxidant content present in spirulina powder was not found in the ethanol extract of spirulina.¹⁵

Spirulina can act as a food, immunomodulatory therapy, antioxidant, and anti-infective. Spirulina supplementation of 2.5% or 5% in *Oncorhynchus mykiss*, was able to increase the secretion of mucin acid goblet cells with sulfomucin and sialomucin content which prevent bacterial infection and other pathogens.²⁸ Spirulina extract is beneficial in increasing the amount of IL-1, NK cell activity, mobilization of immune cells, and the production of IgM, IgA, and IgE antibodies.²⁵ C phycocyanin can decrease proinflammatory cytokines (TNF- α , IL-6, MMP-3, NO, glycosaminoglycans sulfate), and down-regulation of mRNA in iNOS, COX-2, TNF- α , and IL-6.²⁹ In *Candida albicans*, administration of spirulina activates monocytes, expresses IL-1 β , TNF- α , and increases IFN-production.²⁶ Phycocyanin as a potent antioxidant effect on the assay 1,1-diphenyl-2-picryl hydralazine (DPPH) and 2,2'-azino-bis (3-ethylbenzothiazoline-6 sulphonic acid) (ABTS), oxidative stress reducing inflammatory disease.²⁸ Spirulina plays an indirect role in anti-inflammatory and antioxidant, especially in TNF- α and IL-1 β .

The difference in the results of protein expression mucin can be influenced by various factors. Changes in protein expression mucin associated with inflammation, for example in TNF- α , NF- κ B, and IL-1 β , as well as their effect on the administration of sambiloto and spirulina. A decrease in Muc-1 in the ileum and colon can be found due to the presence of microbiota. Microflora can regulate distribution mucin intracellular with cytoskeletal proteins. In addition, the presence of zinc consumption can have an effect on increasing goblet cells synergistically with an increase in Muc-1 mRNA. The microbiota that ferments carbohydrates to butyrate can increase Muc-1.³⁰

The limitation in this study is the process of assessing the mechanism of action associated with decreased expression of Muc-1 by sambiloto and spirulina. These results differ from other studies in which these herbs can increase mucus production. This could be influenced by factors not assessed in this study such as inflammatory cytokine activity, such as TNF- α , NF- κ B, and IL-1 β , microbiota activity in the mouse colon, and diet.

CONCLUSION

- The combination of sambiloto extract (200 mg / Kg/BW) and spirulina powder (130 mg / kgBW) in reducing the expression of Muc-1 protein in the colon media of mice infected with *Plasmodium berghei* ANKA is better and more effective than sambiloto monotherapy (200 mg / Kg/BW) or control negative (CMC 0.5%).
- The combination of sambiloto with spirulina powder had better effectiveness in reducing Muc-1 expression in the colon media of mice infected with *Plasmodium berghei* ANKA than the combination of sambiloto and spirulina extract.

SUGGESTION

- This study can be the basis for other studies to develop the effect of sambiloto extract and spirulina powder on the expression activity of the proinflammatory cytokines TNF- α , NF- κ B, and IL-1 β , as well as taking into account the influencing factors such as diet and microbiota related to decreased expression of Muc-1 protein in the colon media of mice infected with *Plasmodium berghei* ANKA.
- A test group for a combination of sambiloto extract with aqueous extract of spirulina compared with a combination of sambiloto extract with powder or ethanol extract of spirulina is needed to see the effectiveness of spirulina antioxidants in reducing Muc-1 protein expression in the colon media of mice infected with *Plasmodium berghei* ANKA.

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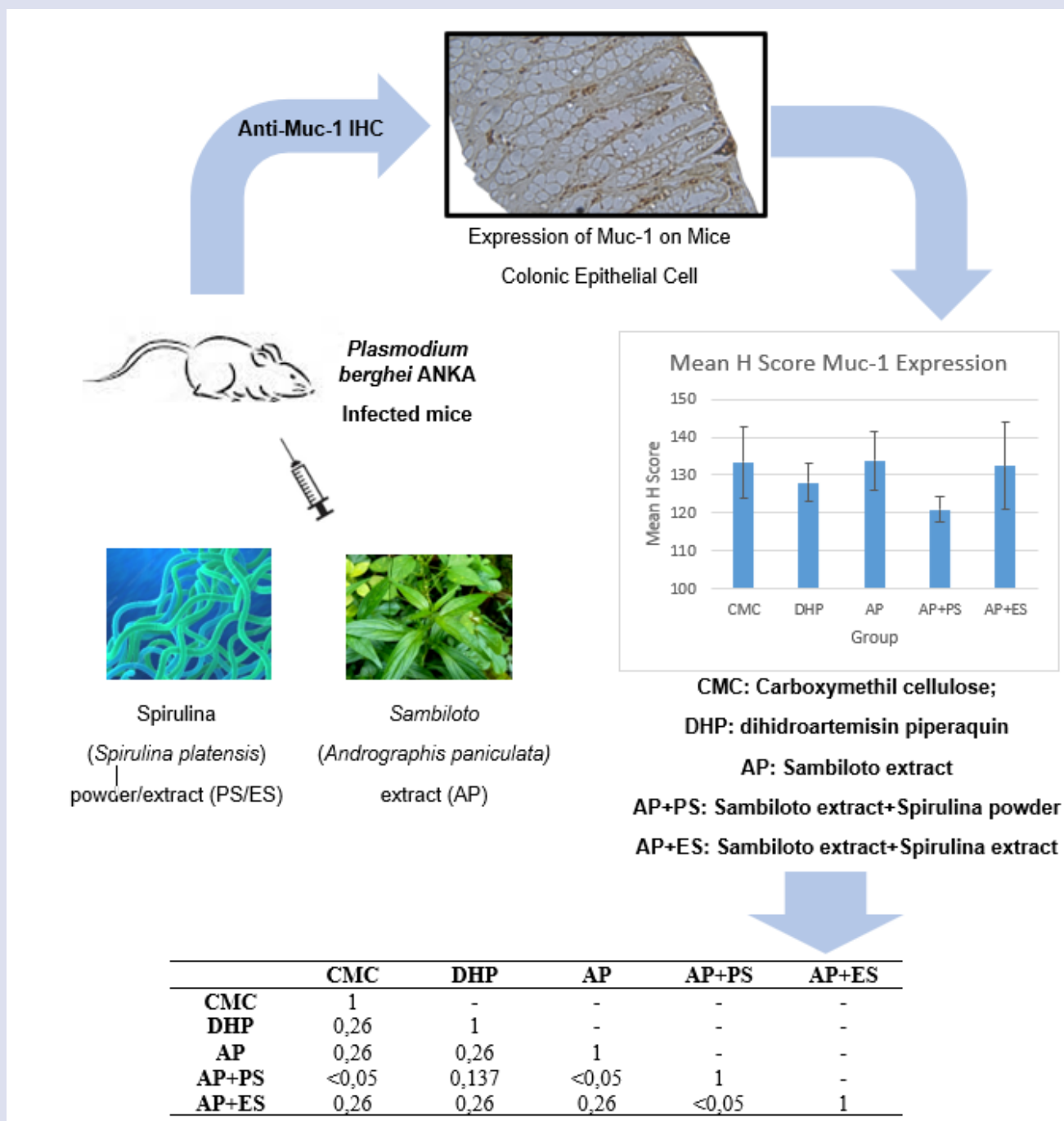
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GRAPHICAL ABSTRACT



ABOUT AUTHORS



Dr. Kusmardi, MSc. is an associate professor at The Anatomic Pathology, and the Doctoral Program in Biomedical Sciences Faculty of Medicine, Universitas Indonesia. He is also the senior researcher at The Drug Development Research Cluster, and The Human Cancer Research Cluster, Indonesia Medical Educational and Research Institute, Jakarta. The major research focus on colorectal and breast cancer, include the potential inhibition of some Indonesian natural medicine on the both carcinogenesis, the identification of normal tissue vs cancer development using some molecular marker and computational model. He wrote the mouse model for breast cancer book, the mouse model for colorectal adjuvant chemopreventive book, and Lunasin: a soybean polypeptide as chemopreventive adjuvant for colon cancer.



Baiqi Nur Hairi is Undergraduate Program Student of Faculty of Medicine, Universitas Indonesia. Research interest in Pharmacology, Herbal Medicine, Public Health.



Nadar Sukri Lubis, M.Sc. is a Lecturer and Reseacher at Parasitology Department, Faculty of Medicine, University of Indonesia. Research Interest in parasitology.



Tri Wahyuni Lestari, MSc, Apt. is a senior researcher at The National Institute of Health Research and Development, Ministry of Health of Indonesia, Jakarta, Indonesia. Research Interest in parasitology and herbal medicine.



Putri Reno Intan, dr Vet is a researcher at The Centre for Research and Development of Biomedical and Basic Health Technology, National Institute of Health Research and Development, Ministry of Health, Jakarta, Indonesia. Research interest: Biomedical Sciences, Pathology, Herbal Medicine and Animal Model

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