



Effect of Ethanol Extract and Ethyl Acetate Fraction of Betel Nut (*Areca catechu* L.) in Colonic Goblet Cells of Mice (*Mus musculus*) Given Orally Infective Egg of *Trichuris muris*

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Abstract

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BACKGROUND: *Trichuris trichiura* is a soil-transmitted parasitic worm commonly found in humid, tropical to subtropical areas, as well as poor sanitation area. These worms are cosmopolitan, especially in tropical and humid countries like Indonesia. This worm infection is more common in developing countries and more in children than adults due to poorer child self-hygiene. Worm disease is one of the common society diseases with 60% of children in Indonesia which are estimated to be affected. One species of *T. trichiura* worm that is often used in research on experimental animals is *Trichuris muris*. The administration of *T. muris* infective eggs can significantly increase the number of goblet cells in intestinal crypts of colon and cecum. Goblet cell hyperplasia will occur after exposure to high-dose *T. muris* (acute/200 infective eggs) in resistant mice. Various studies on the development of natural and traditional materials have been developing in the past few years to see the effects of betel nut on the number of goblet cells in the large intestine.

AIM: The aim of this research is to determine the effect of ethanol extract and the ethyl acetate fraction of betel nut on the change in the number of goblet cells in male mice given orally *T. muris* infective eggs.

METHODS: This study was an experimental study with a post-test only control group design in male mice (*Mus musculus*) which were given 200 infective eggs of *T. muris*. The study sample consisted of 70 mice divided into seven groups. Doses of the ethanol extract of betel nut are 100 mg/kg b.w. and 150 mg/kg b.w., and doses of ethyl acetate fraction are 100 mg/kg b.w. and 150 mg/kg b.w. The independent variable was ethanol extract and ethyl acetate fraction of betel nut. The dependent variable is the number of goblet cells. Mean differences in groups were tested by Mann-Whitney.

RESULTS: Statistical analysis showed a significant difference of $p < 0.05$ in the colonic crypts and cecum. In Group K (+), there was no increase in the number of goblet cells (54.2000 ± 30.54864) compared to Group K (-) (79.0750 ± 11.79221) in the colon. There was an increase in goblet cells in the 150 mg/kg b.w. of ethanol extract group (64.1000 ± 29.50381), the 150 mg/kg b.w. of ethyl acetate fraction group (56.2250 ± 22.11035), and the 1 mg/20 g of albendazole group (68.3750 ± 11.30956) in the colon. Likewise, there was an increase in the number of goblet cells in the cecum. It can be concluded that 150 mg/kg b.w. of the ethanol extract of betel nut can effectively increase the number of goblet cells in the colon and cecum.

CONCLUSION: Ethanol extract of betel nut 150 mg / kg of weight can effectively increase the number of goblet cells in the colon and caecum.

Introduction

Worm infections, especially infection that is transmitted through worm's egg-contaminated soil (soil-transmitted helminths), are considered as neglected diseases. Intestinal worm infections or intestinal helminthiasis is a chronic condition with no obvious clinical symptoms nor visible short-term effects, yet the attention to this disease is lacking. *Trichuris trichiura* infection is one of the most difficult conditions to cured compare to other worm infections. The infection caused by *Ascaris lumbricoides* and hookworm, for example, could be cured with 1 day medication treatment. *T. trichiura* infection, on the other side, requires medication for 3 days in a row to show some improvement [1].

Intestinal worms are always well adapted to their environment. As a consequence, most people with the infection show no symptoms and are uninformed about existence. *T. trichiura* is actually showing biological and antigenic similarity with *Trichuris muris*, which is a natural pathogen in mice. The infective eggs, that are given orally, will hatch distally in the small intestine and attack the intestinal epithelial cells that line the crypts of the cecum and proximal colon.

Worms may stimulate more than 1 immunological defense mechanism, both humoral and cellular immune responses. The greater the size of the worm, the more types of antigens that generate an immune response. Immunoglobulin E (IgE) production increases in the cases of worm infections. The eosinophil cells that play a role in killing worms in

the tissue are stimulated by the activation of eosinophil chemotaxis factor (ECF)-A mast cell degranulation (ECF). The body's immune response to the worms will then stimulate the formation of antigens. Antigen-presenting cells (APCs) can stimulate Th0 so that the immune response develops toward Th2.

Immunity arising from the helminthiasis can change the morphology and number of cells, depending on mucosal induction as well as the mechanism of mucus production by goblet cells. Goblet cells are the important elements of the immune response to *T. muris*. Goblet cells in the intestinal epithelium produce mucus as an immune defense to respond inflammation in the digestive tract. The more inflammatory cells, the more mucus produced by goblet cells as a defense cell.

In the past few years, potential medicinal plants as one of the alternatives to minimize side effects and to treat intestinal infection have been developed. Betel nut of *Areca catechu* L. (*A. catechu* L.) has been known as anthelmintic [2]. Betel nut can act as anthelmintic and anti-inflammatory against worm parasitic infections. Betel nuts contain alkaloid compounds (are coline, arecolidine, arecaine, guvacoline, guvacine, and isoguvacine), condensed, and hydrolyzed tannins, flavonoids (flavones), enolic compounds, gallic acid, sap, lignin, evaporated and non-evaporating oils, and salts.

This study discusses the changes in the number of goblet cells in the large intestine of mice (*Mus musculus*) that are fed with *T. muris* infective eggs treated with ethanol extract as well as with the ethyl acetate fraction of betel nut. The examination of the number of goblet cells is a rare study. This study aims to determine the effect of ethanol extract and ethyl acetate fraction of betel nut seeds on the number of intestinal goblet cells in male mice that were given *T. muris* infective eggs through oral administration.

Materials and Methods

Experimental design

This study is an experimental study with a post-test only control group design, using male mice (*M. musculus*) fed with *T. muris* infective eggs. The inclusion criteria were as follows: (1) Male mice (*M. musculus*) age 2 months old, (2) mice are in a healthy/active condition, (3) mice weigh are between 20 and 30 g, and (4) mice were infected with a dose of 200 *T. muris* infective eggs. The exclusion criterion was male mice that died during the study. Furthermore, the independent variable examined in this study is the dose of ethanol extract (150 mg/kg b.w. and 100 mg/kg b.w.) and ethyl acetate fraction (150 mg/kg b.w. and 100 mg/kg b.w.) of betel nut (*A. catechu* L.) and albendazole 1 mg/ 20 g. The dependent variable is the number of goblet cells.

Experimental groups

The sample of this study consisted of 70 mice which were divided into seven groups, in which each group contained 10 mice. The negative control group was given no treatment other than *ad libitum* food and drink in their cages as common practice. Meanwhile, the positive control group was given 200 eggs *T. muris* infective (acute infection). Group P1 was the treatment group administered with ethanol extract of betel nut 100 mg/kg b.w, Group P2 was administered with ethanol extract of betel nut 150 mg/kg b.w, Group P3 was administered with ethyl acetate fraction of betel nut 100 mg/kg b.w, Group P4 was administered with ethyl acetate fraction of betel nut 150 mg/kg b.w, and Group P5 was administered with albendazole 1 mg/20 g.

Identification of betel nut samples

Researchers have identified the sample in the Indonesian Institute of Sciences laboratory.

Preparation of ethanol extract of betel nut

The sample used was 10 kg of betel nut taken from the areca nut plantation in the Rantau Prapat area of Medan. The samples were cleaned from dirt and separated from the mesocarp (wet sortation) by washing them with running water until clean. The nuts were dried in an oven with a temperature of 50°C. The dried nuts were then ground to simplicia powder, sifted, and stored in a clean and tightly closed container. The powder was macerated using 96% ethanol solvent. A total of 1000 g of simplicia powder was put into a container and incorporated with 75 parts of 96% ethanol (4.2 L). The container was covered protected from light and left for 3 days with occasional stirring. After the following 3 days, the juice was separated from the waste residual.

The residual was then again blended with 75 parts of 96% ethanol (4.5 L), stirred, and cleaned to obtain 100 parts of the whole juice. The container was closed, kept at room temperature, and protected from light for 2 days. The precipitate was then separated thus a liquid extract was obtained. Thereafter, the liquid extract was evaporated using a rotary evaporator at a temperature of 30–40°C and then concentrated using a freeze dryer at a temperature of -4°C to obtain a dry extract of betel nut.

Preparation of ethyl acetate fraction of betel nut

The fraction was produced by liquid extraction using ethyl acetate solvent. A total of 10 g of ethanol extract and 100 ml of distilled water were homogenized, brought into a separating funnel, and allowed to stand for a while. Ethyl acetate fraction (50 ml) was added into

the tunnel, shaken, allowed to stand until the layer of ethyl acetate fraction formed. Fractionation was carried out until the ethyl acetate layer was pure.

Infective eggs administration

Mice in each treatment group were orally induced with 200 *T. muris* infective eggs, except the negative control group, and were left for 30 days. On day 31st–33rd, the positive control group was vacated from any treatment. On day 37th, mice were sacrificed.

Identification of alkaloids, flavonoids, and tannins

Alkaloids

Reagents used for the identification of alkaloids were Bouchardat's reagent, Dragendorff's reagent, Mayer's reagent, and Wagner's reagent. A sufficient amount of samples was placed into the tube and mixed with each reagent. One different test tube was used for each test. The positive Bouchardat test would be shown through the formation of chocolate deposits while the formation of brick red deposits would be the positive sign of the Dragendorff's test. Mayer's reagent positive test would be shown by the formation of yellowish-white deposits. Meanwhile, positive Wagner's reagent test would be shown by the formation of chocolate deposits.

Flavonoids

Flavonoid test was carried out using FeCl_3 , Mg/HCl (p), H_2SO_4 (p), and NaOH 10% reagents. Each test involved an adequate amount of sample placed in a test tube and mixed with each reagent. A positive FeCl_3 test would be shown by the formation of black colloids while positive test Mg/HCl would be shown by the pink solution. The H_2SO_4 test would show positive results with the formation of a yellowish-orange solution. The NaOH 10% test would show the positive result with the formation of a blue-violet solution.

Tannins

The tannins test was carried out by dissolving the sample extract into methanol until the sample was completely submerged. Then, add 2–3 drops of 1% FeCl_3 solution. Positive results are indicated by the formation of bluish-black or green.

Analysis of the number of intestinal goblet cells

The sample used in this study was colon and cecum. Then, each part is divided. Each part of the colon and cecum is cut 1 cm long, then washed using 5% formaldehyde so that the remaining dirt is removed

and given a fixative solution of 10% formaldehyde. After that, histopathological preparations are made and stained with hematoxylin and eosin. The calculated parameter is the number of goblet cells per 1000 absorptive cells in the crypto colon and cecum [3].

Hematoxylin-Eosin (HE) Staining

The exploration of the large intestine was carried out, especially to the proximal colon and cecum. The number of goblet cells was calculated through the examination method of HE staining (Figure 1). The staining procedure used in this research followed a basic protocol of dewaxing, dehydration, hematoxylin, differentiation, bluing, eosin, dehydration, clearing, and coverslipping [4].

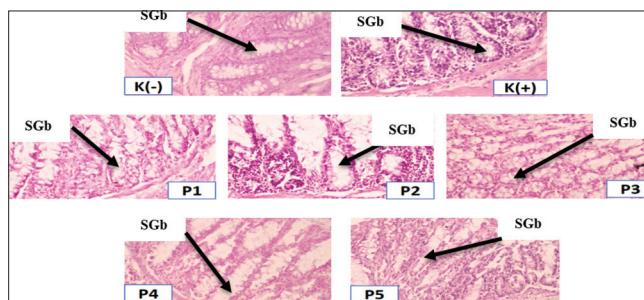


Figure 1: Histopathology of the large intestine (colon proximal) in the male mice on the 37th day after oral administration of 200 infective eggs of *Trichuris muris*. Samples were taken by longitudinal cuts

Results

Analysis of betel nuts identification

The result shows that the betel nut used is identified as the seed of *A. catechu* L., species member of the family *Arecaceae* (Table 1).

Table 1: Identification or determination of betel nut (*Areca catechu* L.)

Name	Species	Family
Betel nut	<i>Areca catechu</i> L.	<i>Arecaceae</i>

Identification of alkaloids, flavonoids, and tannins in ethanol extract and ethyl acetate fraction of betel nut is presented in Tables 2-4.

Table 2: Identification of alkaloids

Sample	Reagent			
	Bouchardat's reagent	Dragendorff's reagent	Mayer's reagent	Wagner's reagent
Ethanol extract of betel nut	+	+++	+++	+++
Ethyl acetate fraction of betel nut	+	++	+++	++

The qualitative identification results show that the ethanol extract of betel nut contains more alkaloids

Table 3: Identification of flavonoids

Sample	Reagent			
	FeCl_3 5%	NaOH 10%	H_2SO_4	$\text{Mg} + \text{HCl}$
Ethanol extract of betel nut	+++	+++	++	+++
Ethyl acetate fraction of betel nut	+++	++	+	+

Table 4: Identification of tannins

Sample	Reagent
	FeCl ₃ 1%
Ethanol extract of betel nut	+++
Ethyl acetate fraction of betel nut	+++

than the ethyl acetate fraction, both in Dragendorff's reagent and Wagner's reagent (Table 2).

The results show that the flavonoids contained in ethanol extract of betel nut (in all reagents used) are greater than in the ethyl acetate fraction, either the one reacted with FeCl₃ 5%, NaOH 10%, H₂SO₄, or Mg + HCl reagents (Table 3).

Based on the identification results in Table 4, the ethanol extract of betel nuts has the same tannins content with the ethyl acetate fraction of betel nuts. The results of the analysis of the number of intestinal goblet cells are presented in Table 5.

Table 5: Number of the goblet cells in the intestinal tunica mucosa of male mice on the 37th day after the oral administration of infective eggs of *Trichuris muris*

Structure of intestinal tunica mucosa (µm)			
Number of goblet cell in	Group	Mean ± SD	p
Crypts of colon	K(-)	79.0750 ± 11.79221	0.000*
	P1	27.6750 ± 10.70374	
	P2	64.1000 ± 29.50381	
	P3	53.6250 ± 18.61759	
	P4	56.2250 ± 22.11035	
Cecum	P5	68.3750 ± 11.30956	0.000*
	K(-)	10.6000 ± 6.46808	
	K(+)	9.5000 ± 2.06492	
	P1	13.5250 ± 6.97461	
	P2	29.0250 ± 7.65257	
	P3	26.6000 ± 4.88791	
	P4	11.4000 ± 4.21670	
	P5	11.1750 ± 4.50779	

*Mann-Whitney

Goblet cells in the colon and cecum showed significant differences in the number of goblet cells ($p < 0.05$).

Discussion

Helminthiasis is a multicellular and complex parasite. It has a long life span and cannot be swallowed by phagocytes so that the host response to helminth infections is usually more complex and stronger [5], [6]. The reaction of the body to fight helminthiasis is characterized by an increase of IgE, tissue eosinophils, mastocyte, and CD4+ cells that produce *T helper 2* (Th2) cells [7]. Worm infection will stimulate APC which will stimulate Th0 so that the immune response develops toward Th2. Recent immune-epidemiological data indicate that the immune response mediated by Th2 cells take part in limiting this worm population [8].

Treatment using medicinal plants is one of the alternatives chosen to minimize the side effects due to the provision of synthetic drugs. Common side effects of anthelmintic medication include stomach pain, diarrhea, and digestive disorders. It may also

cause nausea and vomiting for some people. The medicinal plants have been known to have anti-worm or anthelmintic properties and used until today. From several studies that have been carried out, betel nut (*A. catechu* L.) is one plant that has anthelmintic properties.

Betel nuts contain alkaloids, such as arecoline (C₈H₁₃NO₂), arecolidine, arecain, guvacoline, guvacine, and isoguvacine [9]. The dominant type of alkaloid found in betel nut which is likely to have an anthelmintic effect is arecoline [10]. Arecoline is toxic to several types of worms and causes temporary paralysis [11]. According to Nonaka *et al.* [12] areca nut seeds contain proanthocyanidin, which is a condensed tannin included in the flavonoid class.

Tannins of the ethanol extract of betel nuts have the anthelmintic ability that works by inhibiting the enzymes as well as damaging the membrane [13]. Inhibition of the work of enzymes can cause the process of digestion metabolism of the worm disrupted. The worms would be lack in nutrients and eventually die due to lack of energy. The ethanol extract of betel nut contains a complex metabolite compound compared to the extract of the betel nut ethyl acetate from the results of the phytochemical ring. The ethyl acetate fraction is an advanced process of ethanol extract.

Worm immunity can cause morphological changes as does the number of cells that depend on mucosal induction including the mechanism of mucus production by goblet cells [14]. Goblet cells are an important element of the immune response to *T. muris* worms [15]. The function of goblet cells in the intestinal epithelium produces mucus as an immune defense from the digestive tract that occurs due to inflammation. The more inflammatory cells, the more mucus produced by goblet cells as a cell defense [16].

Phytochemical screening results of the ethanol extract of betel nut that the flavonoids contained in it have a role to inhibit many oxidation reactions that arise due to damage to *T. muris* worms, both enzymatically and non-enzymatically through the role of increasing goblet cells in the worm removal process. This activity can disrupt metabolism and homeostasis in the worm's body so that the worm will die [17], [18].

This study shows that there is a significant difference in the number of goblet cells in the colon and cecum between each group of mice ($p < 0.05$) (Table 5). However, the K (+) group showed no increase in the number of goblet cells (54.2000 ± 30.54864) compared to the K (-) group (79.0750 ± 11.79221) and other groups (Table 5). It indicates that mice have an immune system that is susceptible to helminth infections and betel nut extract may help increase goblet cells in the worm expulsion process.

The administration of *T. muris* infective eggs can significantly increase the number of goblet cells in the intestinal crypts of colon and cecum. It has been

informed that goblet cell hyperplasia will occur after exposure to high-dose *T. muris* (acute/200 infective eggs) in resistant mice [15].

Goblet cells are the main producer of mucus, which form the important innate defense element in the gastrointestinal tract. Goblet cells and their products are important elements of the host immune response to *T. muris* and appear to be crucial for effective cleansing of parasites from the intestine [15]. Goblet cells secrete resistin-like molecules β (RELM- β). RELM- β is indispensable for spontaneous worm expulsion [19]. In this study, there were mice that were resistant and susceptible, which mean that some mice have a role in the exclusion of worms in the “weep and sweep” response.

Goblet cells are the main producers of mucin and form an important innate defense element in the gastrointestinal tract. Goblet cells and their products are important elements of the host immune response to *T. muris* and appear to be indispensable for effective cleansing of parasites from the intestine [15]. Goblet cells secrete RELM- β . RELM- β is indispensable for spontaneous worm expulsion [19].

Epithelial cell replacement is the main mechanism of *T. muris* expulsion. This model of epithelial cell replacement is called an epithelial escalator. Epithelial cells move from the base of the crypts (proliferation zone) to the top of the villi (release zone). As a result, worms attached to the epithelial layer are directed to move toward the lumen where the parasite and epithelium are released. The difference in epithelial cell replacement between susceptible and resistant mice is due to differences in the immune response and its cytokine profile. Th1 and Th2 responses are, respectively, associated with a decrease and increase in regulation of epithelial turnover speed [15]. Phytochemical screening of the ethanol extract of betel nut contains flavonoids and alkaloids which are more in terms of qualitative inspection compared to ethyl acetate fraction.

The results of the examination of the number of goblet cells (Table 5) show the effect of a higher number of goblet cells on ethanol extract 150 mg/kg b.w. (P2) compared to the ethyl acetate fraction 150 mg/kg b.w. likewise with the ethanol extract dose of 100 mg/kg b.w. and ethyl acetate fraction of 100 mg/kg b.w. This study shows that the treatment of ethanol extract of betel nut 150 mg/kg b.w can increase the number of goblet cells in worm expulsion compared to the other dose 100 mg/kg b.w, the ethyl acetate fraction 100 mg/kg b.w and 150 mg/kg b.w, as well as compared to albendazole 1 mg/20 g. The difference in the number of goblet cells (Table 5) in the colon organ shows significant results between the negative control groups and positive controls, P1, P2, P3, P4, and P5. Likewise, the cecum organs are examined.

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