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Research Article

The Expression of Immunohistochemistry IL-10 And IL-18 On Helminth-Infected Mice Treated With The Extract Of Areca catechu L.

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ABSTRACT
Research conducted on T. muris worm infections has shown us how the immune system induces parasitic expulsion so that it can be applied more broadly to the development of the latest therapeutic STH infections. Research and development of natural and traditional ingredients for alternative anthelmintic drugs has been carried out as an anti-inflammatory and to prevent chronic infections in the colon. One of the alternative medicinal plants chosen is Areca catechu L. (A. catechu L.) seeds. This study aims to determine the effectiveness of ethanol extract and ethyl acetate fraction of areca nut on immunohistochemistry (IHC) IL-10 and IL-18 levels in the colon of male mice orally treated with T. muris infective eggs.This study is an experimental study with a post-test only control group design in male mice (Mus musculus) given 200 T. muris infective eggs. The treatment groups are the administration of 100 mg/ kg BW and 150 mg/ kg BW of ethanol extract of areca nut, as well as 100 mg/kg BW and 150 mg/kg BW ethyl acetate fraction. Each treatment was tested for IL-18 and IL-10 IHC levels.The IHC concentrations of IL-9 and IL-18 of colon tissue showed significant differences in 7 groups ($p<0.05$). Meanwhile, IL-10 IHC concentration showed no significant difference. Ethanol extract of areca nut with a dose of 150 mg/kg b.w. showed a more effective result than the ethyl acetate fraction of 150 mg/kg b.w. and 100 mg/kg b.w.Apart from being anthelmintic for T. muris infections, it is concluded that the ethanol extract of areca nut can be used as an anti-inflammation. This makes the use of the ethanol extract of areca nut as one of the alternative treatment options.

Keywords: Anthelmintic, Areca catechu, interleukin, Trichuris muris, worm parasites

INTRODUCTION

Intestinal helminth parasitic infections, especially in mice, have contributed significantly to the advancement of immune responses, which determine the resistance and susceptibility toward diseases. Researches conducted on T. muris worm infections have shown how the immune system could induce parasitic expulsion and so that it applies to the development of new therapeutic soil-transmitted helminthiasis (STH) infections.[1],[2],[3]

Trichuris muris worm is a natural pathogen in mice. It is biologically and antigenically similar to the T. trichiura species that infected humans and livestock. These worm infections have caused significant morbidity and health problems, especially in developing countries. Although the research is quite extensive, the role of the immune system in the infection process is not yet fully understood. As a consequence, the development of antiparasitic therapy is obstructed.[2]

Through the study of T. muris helminth infections, the role of cytokines Interleukin-18 (IL-18) and IL-10 have been discovered. Blood serum IL-18 cytokines play an essential role in the occurrence of chronic gastrointestinal disturbances, while IL-10 plays a role in maintaining colon defense function (colon barrier). If IL-10 deficiency develops, the purpose of the colon barrier will be disrupted, and chronic diarrhea or Trichuris Dysentery Syndrome may occur[3]. The IL-10 produced by Th2 cells causes suppression of inflammatory mediator production. The increasing level of IL-10 is known as an important mediator in host defense against intestinal helminth investment.[4],[5],[6] The expulsion mechanism of T. muris worm infections in the

intestine is shown through the increased epithelial cell turnover, the mucin production by goblet cells, and the contractility of intestine smooth muscle. It, therefore, potentially causes changes in intestine structure.[2]

In recent decades, various research has been developed for the development of natural ingredients for alternative anthelmintic medicines. The potential medicinal plant chosen to reduce side effects and treat intestinal helminth infections is areca palm or Areca catechu L.[7] Areca catechu has many benefits, such as anti-inflammatory, anthelmintic, treatment of diarrhea, treatment of phlegm cough, and some other beneficial use. The seed or nut is part that mostly used for medicinal purposes as it contains alkaloid compounds (arecoline, arecolidine, arecain, guvacoline, guvacine, and isoguvacine), condensed and hydrolyzed tannins, flavonoids (flavones), enolic compounds, gallic acid, gum, lignin, oil and isoguvacine, evaporating and non-evaporating oil, and salt. Areca nut also contains proanthocyanidin, which is a condensed tannin that belonged to the flavonoid class. The proanthocyanidin is known to have anti-inflammatory effects.[8]

Given the many benefits of areca nuts explained above and the demand for scientific information related to the composition, the study of the anthelmintic and anti-inflammatory properties of areca nuts are important. Areca nut is expected to be a natural alternative drug to be developed. As areca nut extract shows to be more efficient as an anti-inflammatory rather than as an anthelmintic, this study aims to investigate the effectiveness of ethanol extract and the ethyl acetate fraction of areca nut on cytokines changes in immunohistochemistry (IHC) IL-10 and IL-18 levels in colon tissue of male mice fed with T. muris infective eggs. Furthermore, it also aims to compare the effectiveness of both extracts.

MATERIALS AND METHOD

Materials

This study is an experimental study with a post-test only control group design in male mice (Mus musculus) treated with T. muris infective eggs. This study was conducted to determine the effectiveness of ethanol extract and the ethyl acetate fraction of areca nut against changes in the levels of cytokines IHC IL-10 and IL-18 colon tissue in male mice orally fed with T. muris infective eggs.

Areca Nut Preparation

About ten kg of areca nut (wet weight) was cleaned from dirt by washing them with running water. It was then dried in an oven with a temperature of 50℃. After being dried, the dried simplicia was powdered and sifted. Powder simplicia was then kept in a clean and tightly closed container.

Preparation of Ethanol Extract

The powder simplicia prepared was macerated using ethanol solvent. Thousand g of simplicia powder was put into the vessel and poured with 75 parts of 96% ethanol (4.2 L). It was then covered and left for three days, protected from light while repeatedly stirring. After three days, the juice was cleaned, and the pulp was squeezed. The flesh was added with 75 parts of 4.5 L liquid (96% ethanol) and then stirred and washed to obtain 100 parts of the whole juice. The vessel was closed, left in a cool place, and protected from light for two days. The precipitate was then separated; thus, a liquid extract was obtained. The extract obtained was evaporated using a rotary evaporator at a temperature of 30 ℃ - 40℃ to get a thick extract. The thick extract was then evaporated again using a freeze dryer at a temperature of -4℃ until the areca nut extract was dried.

Preparation of Trichus muris infective egg induction

The two months old male mice (Mus musculus) with the healthy condition and body weight of 20-30 g were induced with a dose of 200 T. muris infective eggs. Egg-induced mice were given ethanol extract of areca nut at a dose of 100-150 mg/kg b.w. and ethyl acetate fraction at a dose of 100-150 mg/kg b.w.

The research sample consisted of 70 mice divided into seven groups. Mice were induced with 200 T. muris infective eggs orally in each group except the negative control group and were left for 30 days. On the 31st day through the 33rd day, the positive control group (treatment) received no treatment. Meanwhile, the P1 group was treated with ethanol extract of areca nut p.o. (orally) 100 mg/kg, group P2 was administered with ethanol extract of betel nut, p.o. 150 mg/kg, P3 group was administered with ethyl acetate fraction of areca nut, p.o. 100 mg/b.w, group P4 was administered with ethyl acetate fraction of areca nut, p.o. 150 mg/kg, and group P5 was administered with albendazole p.o. 1 mg/20 g. On day 37, mice were taken blood through the cardiac puncture and sacrificed (sacrificed). Examination of the intensity of worms is done by taking feces of mice in the large intestine and examined under a microscope by the Kato-Katz method. The analysis was carried out by immunohistochemistry (IHC) test of Interleukin-10 and Interleukin-18 in colonic tissue.

Statistical Data Analysis

The collected data were analyzed with the inferential analysis. It was started with the normality test, the Saphiro Wilk test, and the homogeneity of the data. If the data was normally distributed and homogeneous, an ANOVA test was performed to check the differences in the expression levels of cytokine IHC IL-10 and IL-18 colon tissue. If the data were not normally distributed, then the Kruskal Wallis test would be used.

Result

Analysis of Areca Seed Identification

The identification or determination of areca nut plants shows that the species used was Areca catechu L., which is the species member of the Arecaceae family.

Phytochemical Screening of Areca Seed Extract

Table 1. Alkaloid identification

Sample	Identification Test		
	Dragendorf	Mayer	Wagner
Ethanol extract of areca seeds	+++	+++	+++
Ethyl acetate areca seeds	++	+++	++

Based on qualitative identification results using Dragendorf and Wagner reagents, the ethanol extract of areca nut contains more alkaloids than ethyl acetate fraction (Table 1). Furthermore, the results of identification of flavonoid compounds are presented in Table 2

Table 2. Flavonoids identification

Sample	Identification Test		
	NaOH 10%	H2SO4	Mg+HCL
Ethanol extract of areca nuts	+++	++	+++
Ethyl acetate areca nuts	++	+	+

The results of the identification of various reagents show that the ethanol extract of areca nut contains more flavonoids than ethyl acetate fraction in 10% NaOH reagents, H2SO4, and Mg + HCl.

Table 3. Tannin identification

Sample	Identification Test
	FeCl3 1%
Ethanol extract of Areca seeds	+++
Ethyl acetate fraction of areca seeds	+++

Based on the identification results in Table 4, the ethanol extract of areca nuts has the same tannin content as the ethyl acetate fraction of areca nuts. Worm infection is still one main problem in developing countries like Indonesia and also one of the most common infections spread in the world. Worm disease is estimated to infect more than 60% of children9,10. Worms are multicellular and complex parasites, have a long-life span, and cannot be swallowed by phagocytes. As a result, the host response to worm infections is usually more complex and stronger11. The reaction of the body to fight worm infection is marked by an increase of immunoglobulin E (IgE), tissue eosinophils, mastocytes, as well as CD4+ cells that produce

helper 2 (Th2) cells12. Worm infection will stimulate Antigen Presenting Cell (APC), which will stimulate Th0 so that the immune response develops towards Th2. Recent immuno-epidemiological data indicate that immune responses mediated by T helper 2 (Th2) cells play a role in limiting this worm population13.

IL-18 is an essential cytokine for the induction of pro-inflammatory response in the intestine. The induction is regulated in the intestinal mucosa in patients with inflammatory bowel disorders such as Crohn's disease. Interestingly, some recent reports stated that IL-18 plays role in encouraging Th2 responses. There was an increased level of IgE, IL-4, and IL-13 after stimulation in vivo or in vitro with IL-18. Therefore, it appeared that IL-18 is a pleiotropic cytokine that may have a different role in the immune response depending on the location and the time of the induction. As cytokine that plays an essential role in T. muris resistance is IL-10 molecules, mice with IL-10 deficiency become more susceptible to T. muris infection and may develop more severe intestinal abnormalities.[14],[15],[16]

Treatment using medicinal plants is one of the alternatives chosen to minimize the side effects of the administration of synthetic drugs.[17] According to several studies, plants with anthelmintic properties have been obtained included areca nuts (Areca catechu L.).[18] The results show that the content of alkaloids and flavonoids in ethanol extract of areca nut was better than the ethyl acetate fraction (Tables 1, 2, and 3). Alkaloids show its anthelmintic effect by causing the worm's body proteins to clump. As a result, the metabolism and homeostasis in the worm's body are disrupted and may later be followed by the death of the parasite.[19] Tannins of the ethanol extract from areca nuts have the anthelmintic ability that can inhibit enzymes and damage the worm's membrane.[20] Inhibition to the work of enzymes can disrupt the digestive, metabolic processes of the worms and cause it to have a lack of nutrients. In the end, the worms will die due to a lack of energy. The mechanism of action of flavonoids as natural antioxidants is by reducing or neutralizing free radicals such as ROS and RNS through inhibition of enzymes that produce superoxide anion radicals such as xanthine oxidase and protein kinase. Flavonoids also reduce cyclooxygenase, lipoxygenase, microsomal monooxygenase, glutathione S-transferase, mitochondria oxidase mitase, and NADH oxidase. These proteins are all involved in the formation of ROS so that they can repair damaged tissue.[11]

Table 4. Immunohistochemical levels of interleukin-10 and interleukin-18 colon

Group	IL-10 Mean±SD	P-Value	IL-18 Mean±SD	P-Value
K(-)	0.2000 ±0.42164	0.38	8.7000 ±0.94868	0.00*
K(+)	1.1000 ±2.84605		1.9000 ±3.75500	
P1	0.4000 ±0.69921		1.0000 ±1.88562	
P2	0.5000 ±1.08012		6.8000 ±3.67575	
P3	0.4000 ±0.84327		1.3000 ±1.94651	
P4	0.2000 ±0.42164		1.3000 ±1.49443	
P5	0.3000 ±0.67495		0.9000 ±2.84605	

*Kruskal Wallis Test (Significance p<0,05)

The results of IL-18 IHC concentration levels show that there was a significant relationship between treatment groups ($p < 0.05$). Meanwhile, IL-10 IHC levels obtained p-value > 0.05 and shows no significant difference.

It is shown clearly in the IHC level (Mean ±SD), in which the positive controls (1.9000 ± 3.75) will decrease quantitative levels compared to P1 groups and increased to P2 groups (6.8000 ± 3.67). This study shows that susceptibility related to Th1 response involves activation of IL-18 and Caspase-1, followed by IL-12 and interferon (IFN)-gamma in the intestine. Mice with IL-18 deficiency are very resistant to chronic T. muris infection. The ethanol extract of areca nut, meanwhile, has a role as an anthelmintic and anti-inflammatory, which can prevent chronic infections.

In the study of Poela and Hanafiah.[21], the anti-inflammatory activity test showed that the three test groups of ethanol extract of areca nut (Areca catechu L.) gave an anti-inflammatory effect. In this study, the optimal dose between the variant doses is 160 mg/200g b.w, which has an inflammation inhibition of 64.19% in the first hour. However, it is not equivalent to the inhibition of inflammation of sodium diclofenac, which is 78.52% in the first hour. On the other side, both doses of 120 mg / 200 g b.w. and 160 mg/ 200 g b.w. showed a faster inhibition of inflammation compared with Na-diclofenac. It is possibly due to the differences in pharmacokinetic properties between proanthocyanidin and sodium diclofenac.

This study tells us that IL-18 pro-inflammatory cytokines are an essential component in the development of chronic gastrointestinal infections. Furthermore, this study also shows that the decrease of IL-18 during T. muris infection causes an immune response to develop in a chronic direction. This is mediated by Th2, which develops towards the T regulator (Treg).

IL-10 IHC results in this study showed no significant differences (Table 4). This can be seen in the gap of mean ± SD, which is in all between groups. As the IL-10 plays a role in maintaining colon defense function (colon barrier), the IL-10 deficiency may cause the barrier function of the colon to get disrupted. It further can cause interference with infection in the colon, with symptoms like diarrhea. Areca nut extract has anti-inflammatory effects in chronic gastrointestinal disorders and intestinal defense. On the other side, the ethanol extract of the areca nut contains alkaloids and flavonoids which have anti-inflammatory activity in tissue damage

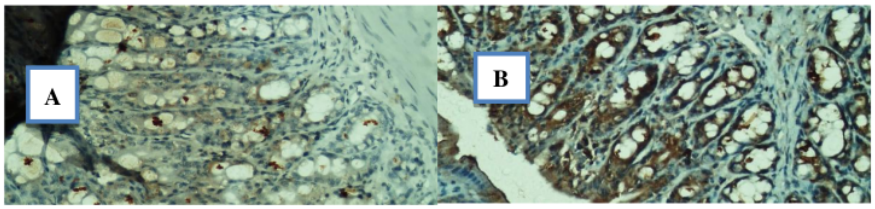

Fig.1. (a) Expression of IHC IL-10 dan (b) expression of IHC IL-18 on 400x.

This study shows that ethanol extract of 150 mg/kg b.w. areca nut has an anti-inflammatory effectivity in preventing chronic infection in the large intestine caused by T. muris infection. It is more effective compared with ethanol extract of 100mg / kg b.w areca nut, 100 mg/kg b.w. areca nut ethanol extract/fraction kg b.w. and albendazole 1mg/ 20g.

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