Short Communication

Antibacterial and anti-inflammatory activities of *Nothopanax scutellarium*, *Moringa oleifera* and *Piper betle* extracts on staphylococcal mastitis animal model

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Abstract

Inappropriate and prolonged administration of antibiotics in mastitis could cause resistance and herbal treatment might could be one alternative treatment. *Nothopanax scutellarium*, *Moringa oleifera*, and *Piper betle* are medicinal plants that contain various active compounds, including antibacterial and anti-inflammatory agents, but their potential in treating mastitis is minimum. The aim of this study was to assess the effectiveness of those plants against mastitis in rabbit model induced by *Staphylococcus aureus*. A total of 25 lactating rabbits (*Oryctolagus cuniculus*) weighing 3.0±0.4 kg were grouped into five groups: healthy control; mastitis control, and three treatment groups (*Nothopanax scutellarium*, *Moringa oleifera*, and *Piper betle*). Except the negative control, all animals were inoculated with 0.15 mL of *S. aureus* containing 1.5x10^7 colony forming unit (CFU)/mL on eight days after giving birth. The extract was administered orally after four hours *Staphylococcus aureus* inoculation at a dose of 50 mg/kg body weight, twice a day for five consecutive days. The number of bacteria in the milk and the level of serum interleukin 6 (IL-6) were measured and histopathological examination of mammary gland tissues were analyzed. The log number of total plate count of *Staphylococcus aureus* indicated that all extract groups had significant lower of bacterial logs compared to mastitis control (all comparisons had $p<0.05$) with the lowest was found in *Piper betle* group, followed by *Nothopanax scutellarium* and *Moringa oleifera* groups. The enzyme-linked immunosorbent assay (ELISA) results showed that all ethanolic extract groups had significantly lower levels of IL-6 compared to the mastitis control (all comparisons had $p<0.05$). The histopathology assessment suggested that extract groups had lower infiltration of inflammatory cells such as lymphocytes and macrophages in alveoli compared to the mastitis control group. In conclusion, all three extracts contained antibacterial and anti-inflammatory activities and *Piper betle* had the most effective in reducing bacterial growth and IL-6 level compared to others.

Keywords: *Nothopanax scutellarium*, *Moringa oleifera*, *Piper betle,* mastitis, interleukin 6

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Introduction

Mastitis is an inflammatory condition characterized by erythema and increased temperature in the breast, accompanied by fever, and in some cases, nausea and vomiting. This condition typically occurs during lactation, with 75-95% of cases arising before the infant reaches three months of age [1, 2]. The primary underlying factors include milk stasis, which leads to infection, as well as the stagnation of breast milk, providing an environment conducive to bacterial growth [1, 3]. *Staphylococcus aureus* is the predominant causative bacterium responsible for mastitis [4, 5]. If left untreated, mastitis can progress to the development of a breast abscess, which represents a complication of the infectious process [6]. In response to the infection, the innate immune system releases interleukin 6 (IL-6), but its overexpression could contribute to pathologic condition in chronic inflammation and autoimmunity [7].

Currently, antibiotics are used to treat this disease, but researchers have investigated herbal plants with antibacterial activities as the alternative [8–10]. Moreover, concerns have been raised about the overuse of antibiotics associated to the emergence of multi-drugs resistance bacteria. In this present study, researchers aimed to investigate the commonly used ethnomedicinal plants in Indonesia, namely *mangkokan* (*Nothopanax scutellarium*), *kelor* (*Moringa oleifera*), and *sirih* (*Piper betle*). In previous studies, these plants have been shown promising antibacterial activities against *S. aurues* [11–13]. However, the activities of these plants against *S. aureus*-associated mastitis in animal model are still underreported.

Methods

Study design

A laboratory experimental study was conducted using five groups of rabbits (*Oryctolagus cuniculus*) comprised of healthy control, mastitis control, and three treatment groups (*N. scutellarium, M. oleifera*, and *P. betle*, respectively). According to the Federer's calculation, the number of rabbits in each group was five which were further randomly assigned. The treatment was performed for five days by administering the extract. Milk, blood, and mammary gland tissue of each rabbit were extracted on the final day 6 hours after the last extract administration. Observations were carried out on the bacterial colonies in the milk, serum IL-6, and histopathology of the mammary gland tissue.

Extract preparation

Five kilograms each of *N. scutellarium*, *M. oleifera*, and *P. betle* leaves were collected and washed with running water to remove dirt and other particles then air-dried and ground into powder. *N. scutellarium* leaves were obtained from Banda Aceh and Aceh Besar, Indonesia. The extraction protocol followed the suggestion from a previous study [14]. Briefly, air-dried powder of each plant (300 g) was macerated with 3 L ethanol 70% for seven days at room temperature. Thereafter, the filtrate was collected and concentrated with a rotary evaporator (Butchi Rotavapor®, Switzerland) to obtain the crude extract of each plant.

Animals

Twenty-five lactating rabbits (*O. cuniculus*) weighing 3.0±0.4 kg was obtained from the Laboratory of Faculty of Veterinary Medicine, Universitas Syiah Kuala, Banda Aceh, Indonesia. The acclimatization process was carried out for seven days. Sick animals during the acclimatization process were excluded from the study. The rabbits were kept in hygienic laboratory-standard cages, receiving food and water *ad libitum*. The animal adaptation and treatment complied to the Ethical Committee procedures.

Bacterium suspension preparation

S. aureus (ATCC, 25923) KS 66215 (Lenexa, KS, USA), and the culture was incubated in a nutrient broth agar at 37°C within 18 hours. Harvested bacteria were diluted in nutrient broth (10^{-1}) and incubated for 2.5 hours until they achieved exponential growth. After incubation, they were centrifuged at 5000 rpm for 10 min and rinsed three times in endotoxin-free phosphate buffered

saline (PBS). The cell pellet was resuspended in PBS at a concentration of 1.5x10^8 colony forming unit (CFU)/mL.

Mastitis infection model and treatment

At eight days after giving birth, the lactating *O. cuniculus* were milked until emptied. They were then inoculated with 0.15 mL of *S. aureus* suspension (1.5x10^7 CFU/mL) at the base of the nipples in positions 3 from left (L3) and right (R3) using a 30-gauge needle. After 4 hours of the inoculation, the extracts were administered orally with nasogastric tube twice a day for five consecutive days. On the final day (day 5), 6 hours after the last administration of extracts, milk and blood samples were collected, and the rabbits were euthanized for mammary gland tissue collection.

Bacteriological examination and total plate count

Upon the collection, the milk sample (50 µL) was diluted 1000 times, where 10 µL of the total solution was spread into mannitol salt agar (MSA). The MSA was incubated for 18–24 hours at 37°C, and the colonies were calculated using total plate count (TPC).

Measurement of inflammatory mediator concentration

The quantity of inflammatory mediator (IL-6) in rabbit sera was measured using a rabbit IL-6 ELISA kit (Cat. No. BZ-08173000-EB, Bioenzy, Jakarta, Indonesia). An approximately 5 mL venous blood was collected from the marginal ear vein and immediately centrifuged for 10 min at 2500 rpm. After centrifugation, the supernatant fluid was collected and stored at -20°C until used. The procedure for measuring the IL-6 concentrations followed the manufacturer's instructions (Bioenzy, Jakarta, Indonesia). The sample (40 µL) was mixed with 10 µL anti-rabbit IL-6 antibody. A total of 50 µL of streptavidin-HRP was used and 50 µL of the stopping solution was added to each well to stop the enzymatic reaction. The absorbance was measured using an ELISA reader at 450 nm and the concentration calculation was performed on the microplate Manager-6 Program (MPM-6; Bio-Rad Laboratories, Inc., California, USA).

Histology examination

Right after its collection, the mammary gland tissue was placed in a 10% buffered neutral formalin solution. The histopathological examination preparations followed the standard protocol [15]. The preparations were stained with hematoxylin and eosin before being observed using a light microscope.

Statistical analysis

Prior to data analysis, homogeneity of variance and normality tests were performed using the Shapiro-Wilk method. Data were then analyzed using ANOVA, followed by Duncan's test on SPSS 26.0 software (IBM, Armok, New York, USA).

Results

Effect of *N. scutellarium*, *M. oleifera*, and *P. betle* extracts on the growth of *S. aureus* in mastitis rabbits

The number of *S. aureus* colonies in the milk of rabbit in each group, based on TPC method, is presented in **Table 1**. The log number of bacteria in the mastitis control was significantly higher as compared to the others (p<0.05). As expected, the healthy control showed the lowest bacterial logs among others. Of the treated samples, those in *P. betle* group had the lowest bacterial colonies.

Effect of *N. scutellarium*, *M. oleifera*, and *P. betle* extracts on IL-6 levels

To determine the effectiveness of the extracts in inhibiting the production of inflammatory mediator, levels of serum IL-6 were measured, where the data are presented in **Table 1**. As compared to the healthy control, rabbits with mastitis had significantly higher level of serum IL-6 at p<0.05. Levels of serum IL-6 in groups treated with the three plant extracts, respectively, were found to be significantly lower than that in mastitis control (p<0.05). No significant

difference was observed between rabbits treated with plant extracts and those in healthy control (p<0.05).

Table 1. Effects of *N. scutellarium, M. oleifera* and *P. betle* leaf extracts on *S. aureus* growth and IL-6 level in mastitis rabbits

Group	Log total plate count (CFU/mL) *	Interleukin 6 (pg/mL) *
Healthy	0.19±0.26 [a]	166.70±7.67 [a]
Mastitis	5.34±0.018 [d]	203.15±35.4 [b]
Nothopanax scutellarium	3.73±1.28 [c]	180.65±1.07 [a]
Moringa oleifera	3.78±0.05 [c]	179.85±3.88 [a]
Piper betle	2.59±0.15 [b]	169.54±9.06 [a]

* Values with different superscript letter are significantly different at p<0.05

Effects of *N. scutellarium*, *M. oleifera*, and *P. betle* extracts on mammary gland histopathology

Hard and swollen mammary glands and yellowish colored milk were observed in rabbits with mastitis. Histopathological microscopic examinations were performed in rabbits infected with *S. aureus* to determine the effectiveness of *N. scutellarium*, *M. oleifera*, and *P. betle* leaf extracts in inhibiting bacterial growth that causes inflammatory reactions. The histopathological examination of healthy control showed intact alveoli and no inflammatory cells on the alveoli (**Figure 1A**) while mastitis control had alveoli filled with lymphocytes and macrophages as well as alveoli hemorrhage (**Figure 1B**). Some alveoli appeared intact but there were also some alveoli containing lymphocytes and macrophages and hemorrhage in animals treated with *N. scutellarium* extract (**Figure 1C**). Similar change was observed in *M. oleifera* group, where some alveoli appeared intact but there was also the presence of lymphocytes, macrophages, and hemorrhage (**Figure 1D**). The mammary gland in *P. betle* group showed many intact alveoli, with only a few lymphocytes, macrophages, and hemorrhages (**Figure 1E**).

Discussion

Findings of the present study reveal that the ethanolic extract of *N. scutellarium*, *M. oleifera*, and *P. betle* leaves could inhibit *S. aureus* bacteria. Moreover, the ethanolic extract of *N. scutellarium*, *M. oleifera*, and *P. betle* leaves also could reduce IL-6 level, which is a pleiotropic cytokine released in response to tissue injury and infection [7, 16]. It is noteworthy that *S. aureus* produces a series of cytolytic proteins. The streptococcal infection damages cell membranes of erythrocyte, epithelial cells, monocytes and T and B lymphocytes by secreting α-hemolysin toxin [17]. This fact was corroborated by our present histopathologic findings indicating the presence of *S. aureus*-induced hemorrhage. In a previous report, histopathologic findings from sheep with *S. aureus*-associated mastitis indicated the presence of hemorrhage-induced stromal expansion and edema in interlobular connective tissue [18]. Histopathologic damages observed in *S. aureus*-infected mammary gland tissue are resulted from the inflammatory reaction concomitant to innate immunity activation [19]. This is particularly evidenced by the phagocytic activity of macrophages observed in *S. aureus*-infected rodents [20, 21].

Herein, the microscopic image of the mammary glands showed the infiltration of inflammatory cells in the alveoli and shrinkage due to the thickening of the interstitial tissue. Further, alveoli of the infected rabbits in positive control group were filled with inflammations and the interstitial tissue boundaries were not visible. On the other hand, treatment using P. betle leaf extract treatment was found to be capable of inhibiting inflammatory cells infiltration. This anti-inflammatory activity is associated with the phytocompounds contained in the extract including luteolin and kaempferol and some other bioactive compounds (such as catechin, epicatechin, rutin, myricetin, quercetin, apigenin, umbelliferon, and naringenin) [22]. In a previous report, luteolin has been found to protect *S. aureus*-infected mammary glands against inflammatory tissue damage in concentration dependent manner [23]. Moreover, flavonoids in the plant extracts may reduce the edema in mastitis and repair tissue damage by promoting cell regeneration [24]. It is worth noting that this is the first study to report the effect of *N. scutellarium* leaf extract on serum IL-6 level. However, as the limitation of this study, the

activities were not tested with extract concentrations variation. Moreover, the total white blood cell examination was not carried out.

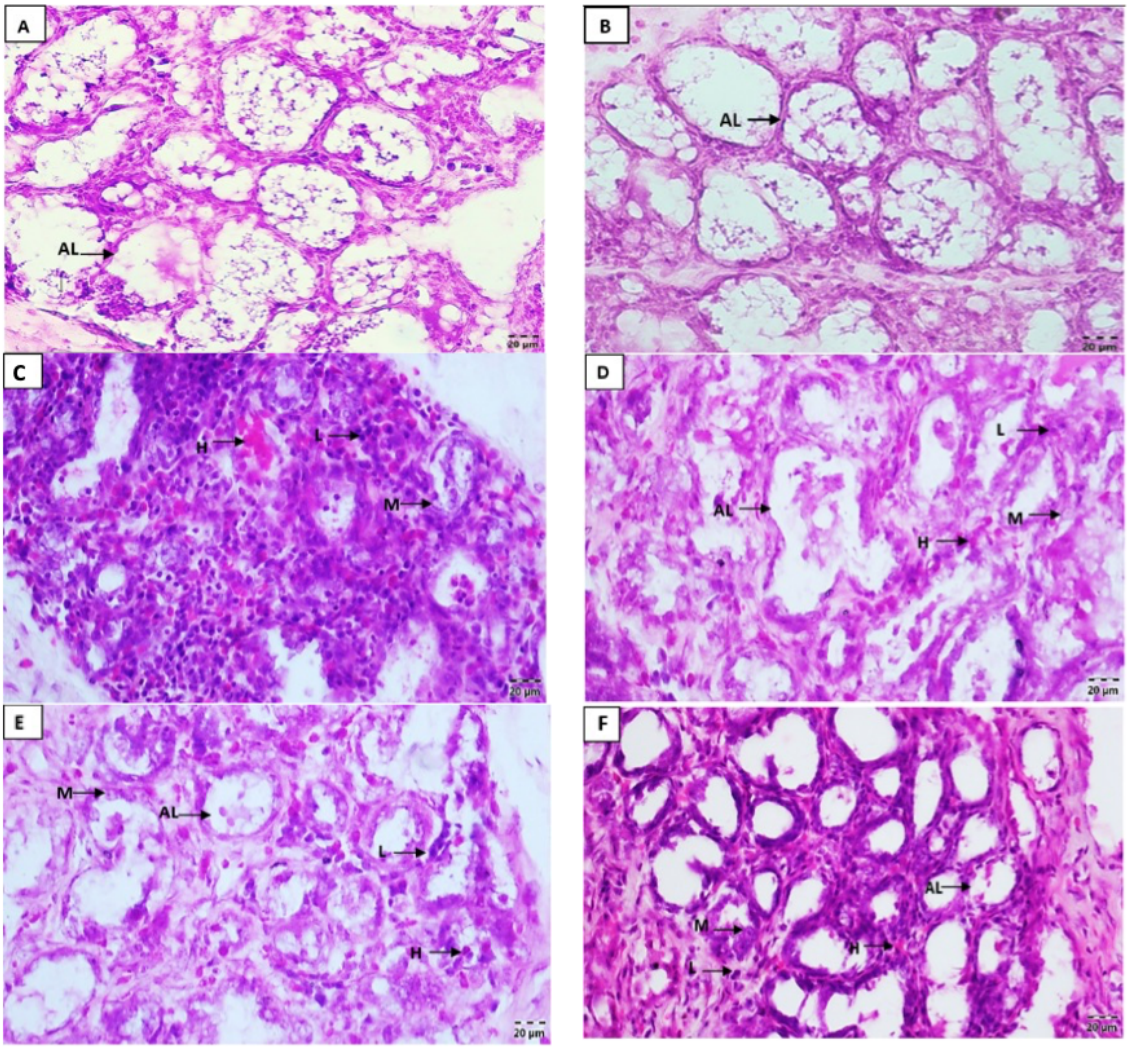

Figure 1. Histopathology of mammary gland of animal models with hematoxylin-eosin (HE) staining at 400x magnification. (A, B) Untreated negative control. (C) Positive control, inoculated with *S. aureus* without any treatment. (D) The animals are inoculated with *S. aureus* and treated with *N. scutellarium* extract. (E) The animals are inoculated with *S. aureus* and treated with *M. oleifera* extract. (F) The animals are inoculated with *S. aureus* and treated with *P. betle* extract. AL: alveoli, H: hemorrhage, L: lymphocyte, and M: macrophage.

Conclusions

Leaf extracts from *P. betle*, *N. scutellarium*, and *M. oleifera*, were revealed to significantly reduce the colonies of mastitis causing *S. aureus* in rabbit, respectively. The single dose observation suggested that *P. betle* leaf extract had significantly higher antibacterial activity against *S. aureus*. The three extracts were also found to restore the dysregulation of IL-6 overexpression that is associated with the minimal damage in the mastitis-affected tissue. Further research should be carried out to determine the median effective dose of the extracts in treating mastitis.

Ethics approval

This study had been approved by Animal Ethics Committee of Faculty of Veterinary Medicine, Universitas Syiah Kuala, Banda Aceh, Indonesia with Ref no: 59/KEPH/VIII/2020.

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Conflict of interest

The authors declare no conflict of interest.

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Underlying data

All data underlying the results can be requested from the corresponding author.

How to cite

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