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Endometritis Control and Enhanced Reproductive Performance in Aceh Cow Using Medicinal Plants

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Abstract: Antibiotic treatment for endometritis has some weaknesses due to its toxicity and unexpected side effects. Therefore, the utilization of plant extract as antimicrobials is an alternative to antibiotics. We aimed to evaluate the efficacy of betel leaf, moringa leaf, and garlic extracts in treating endometritis in Aceh cows. We used nine Aceh cows at 3-5 years old, 150-250 kg body weight, and were diagnosed with endometritis through the White Side Test (WST). All cows were injected with 25 mg Prostaglandin (PGF2α) hormone and cervical mucus was collected 8-12 h post-estrus onset. A 20 mL of red betel leaf, moringa leaf, and garlic extracts with a concentration of 40% was administered every 24 h for 7 days. The extract application was carried out using an insemination gun inserted into the uterus. Post-treatment, the cows were re-synchronized with PGF2α and artificially inseminated after showing symptoms of estrus. Pregnancy was diagnosed using rectal palpation 60-90 days post-insemination. WST score data and total bacterial counts were analyzed using the Wilcoxon test, whereas the reproductive performance data were reported descriptively. The results showed that the administration of betel leaf, moringa leaf, and garlic extracts tended to reduce the WST scores and bacterial counts. The successful pregnancy rates in groups treated with betel leaf extract, moringa leaf, and garlic extract were 66.6, 66.6, and 0.0%, respectively. The application of red betel leaf extract and moringa leaf could reduce the level of endometritis and increase the pregnancy rates in Aceh cows.

Keywords: Aceh Cows, Bacterial Colony, Endometritis, Medicinal Plant, Pregnancy Rate

Introduction

Subclinical endometritis is one of the important etiologic factors of breeding disorders in cattle (Janowski *et al*., 2013). During the reproductive period, the uterus can be exposed to infection, especially during mating and post-parturition (Sheldon *et al*., 2020). The uterine infection could be attributed to microorganisms from the posterior genital organs (vulva, vagina, and cervix) or the environment inside the uterus (Sheldon and Owens, 2017). Thasmi *et al*. (2021) reported that 100% of Aceh cattle with breeding disorders were due to uterine infection.

Pathogenic microorganisms isolated from bovine endometritis are commonly environmental and opportunistic (Nath *et al*., 2014). Generally, a bacterial infection of the uterus can cause endometritis (Carneiro *et al*., 2016), failure of pregnancy, and infertility in cattle (Sheldon and Owens, 2017). It is suspected that an abnormal uterine environment contributes to pregnancy failure in Aceh cattle.

Various therapies to treat endometritis have been carried out, such as prostaglandins, antibiotics (Mido *et al*., 2016; Scenzi, 2016), and Lugol (Ahmed and Elsheikh, 2014; Alyasiri *et al*., 2015; Sutriana *et al*., 2021). Giving 2% lugol can enhance pregnancy rates in Aceh cattle with endometritis

(Sutriana *et al*., 2021). Due to the toxicity, resistance build-up, and side effects of antibiotics, the use of plant extracts and herbs as immunomodulators and antimicrobials is becoming popular (Bhardwaz *et al*., 2018). The use of medicinal plants for the treatment of endometritis and endometriosis in animal models has been studied previously (Wardani *et al*., 2017; Hafsari and Pujiastutik, 2018; Sharma *et al*., 2018; Bina *et al*., 2019). Treatment and control of endometritis in Aceh cattle had been carried out by Rosmaidar *et al.* (2021) using red betel leaf and moringa leaf extract. However, the study was limited to analyzing the decrease in endometritis levels using the White Side Test (WST). In this study, apart from the WST test, total bacterial colony counts and reproductive performance were also observed after treatment. In addition, the plant extracts used in this study were prepared by a subsequent maceration process, while Rosmaidar *et al.* (2021) used a simple extraction process using maceration with ethanol. Therefore, the purpose of this study was to evaluate the efficacy of betel leaf, moringa leaf, and garlic extracts as therapy for endometritis in Aceh cows.

Materials and Methods

Medicinal Plants Extraction

Before extract preparation, the clean red betel leaf, moringa leaves, and garlic were oven-dried at a temperature of 60°C for 48 h. The dry samples were crushed using a blender. The fine powder of each sample was macerated using a multilevel maceration with three solvents: N-hexane, ethyl acetate, and ethanol 96% with a ratio of 1:5 (Dima *et al*., 2016).

The immersion of red betel leaf, moringa leaf, and garlic powder in each solvent was carried out three times for 24 h each (Bachri *et al*., 2015). The extraction process was initiated by soaking red betel leaf, moringa leaf, and garlic powder in n-hexane, followed by ethyl acetate and 96% ethanol. The extracts were then evaporated using a vacuum rotary evaporator (Buchi, R-300) at 150 rpm. The evaporation of n-hexane and ethyl acetate was carried out at 50°C while 96% of ethanol was at 70°C (Srikandi *et al*., 2020). In this study, only the ethanol fraction of betel leaf, moringa leaf, and garlic was used for treating endometritis in Aceh cows.

Experimental Animals

This study used nine female Aceh cows diagnosed with repeat breeding (cows that had been bred >3 times but failed to become pregnant), they were 3-5 years old and 150-250 kg. All cows were synchronized using prostaglandin hormone injected intramuscularly at 25 mg (lutalysetm, Pharmacia and Upjohn Company, Pfizer Inc.) with a single injection and multiple injection patterns. The detection of estrus was performed in the morning (08.00 GMT +7) and afternoon (16.00 GMT +7) for 30 min. Cows were considered in estrus with signs of reddish, swollen vulva and secreted clear cervical mucus.

Cervical mucus collection was performed 8-12 h post-estrus onset to diagnose endometritis using the WST after Bhat *et al*. (2014). The color intensity was observed to determine the endometritis level. In normal cows, the solution was cloudy or colorless (score zero); Mild infection was light yellow (1) Moderate infection was yellow (2) Heavy infection was dark yellow (3).

Upon completion of the WST, the cows with endometritis were grouped into three treatment groups of three cows each. The C1 cows were treated with red betel leaf extract, C2 with moringa leaf, and C3 with garlic extract. The 20 mL of each extract 40% was administered every 24 h for seven days using an insemination gun into the uterus. Each extract was then sprayed with a disposable syringe through an AI pipette. On day 8th post-treatment, the endometritis condition was examined using WST and followed by estrus synchronization using prostaglandin hormone.

Total Bacterial Colonies Count

The bacterial count from the uterus was carried out twice (pre and post-treatment). Samples were collected from the uterine canal using a sterile cotton swab attached to the tip of the AI gun and covered with a sterile plastic sheath and an outer plastic sheath. The gun was inserted into the vagina. To avoid contamination of the vagina, the plastic sheath was directed to the cervix. At the base of the cervix, the AI gun was pushed out of the plastic sheath until it reached the inside of the uterus.

Inside the uterus, the sterile cotton swab was pushed out of the plastic sheath, and the endometrial lining was swabbed. The cotton swab was pulled back into the plastic sheath and removed from the uterus. Swabs were put into Nutrient Broth (NB) media at 37°C/24 h. The colonies were counted using the Total Plate Count method as described by Yunita *et al*., (2015). A total of 18 swab samples were used for the bacteriological treatment (6 samples per treatment).

Artificial Insemination and Pregnancy Examination

Following estrus synchronization on day 8th post-therapy, artificial insemination was performed 12 h after the onset of estrus. A pregnancy examination was carried out using rectal palpation 60-90 days post-insemination.

Data Analysis

WST score data and total bacterial counts were analyzed using the Wilcoxon test, whereas data on reproductive performance which included the percentage of cure, percentage of estrus, and percentage of pregnancy were reported descriptively. Data analysis was carried out using SPPS 24 for Windows.

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Results

The WST was carried out to determine the color change of cervical mucus. A positive indicator of a cow experiencing endometritis is when the cloudy cervical mucus color turns yellow. The WST was performed on cervical mucus obtained from nine cows pre- and post-treatment. In clinical or subclinical endometritis, cervical mucus has an increase in the number of leukocytes which causes a color change. This color change occurs due to the reaction between ribonucleic acid in the white blood cell nucleus with 5% NaOH (Gupta *et al*., 2011). The color change in WST values pre- and post-treatment is presented in Fig. 1.

The total bacterial counts in cervical mucus pre- and post-treated showed similar trends as observed in WST (Fig. 2). The total bacterial counts decreased in the cervical mucus post-treatment. Statistically, the administration of ethanol extract from red betel leaf, moringa leaf, and garlic extract pre-and post-treatment did not show a significant difference, however, the WST scores tended to reduce numerically (Table 1). The reduction in total bacterial counts in C1, C2, and C3 were 32.5, 54.1 and 70.6%, respectively (Table 1).

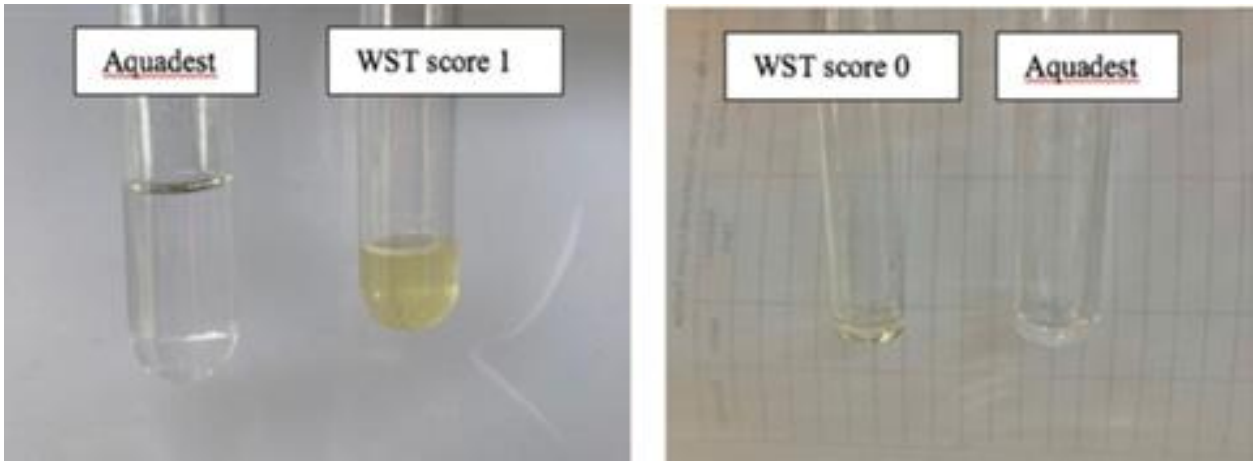

Fig. 1: Changes in WST score of cervical mucus in aceh cow with endometritis pre- (left, WST score 1) and post-treatment (right, WST score 0)

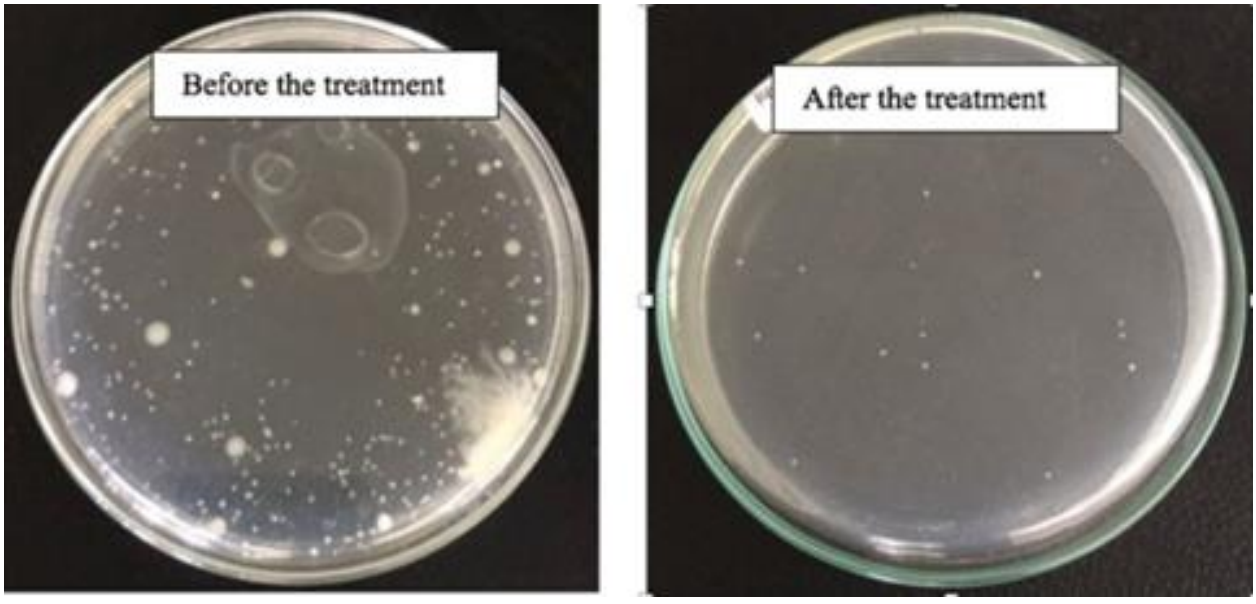

Fig. 2: Total bacterial counts of cervical mucus in aceh cattle with endometritis pre- (left, WST score 1) and post-treatment (right, WST score 0)

Table 1: The average white side test scores and total bacterial counts (± SE) in endometritis cows pre-and post-treatment with red betel leaf extract, moringa leaf extract and garlic extract

	White side test score			Total bacterial counts (CFU/mL)		
Treatments	Pre-treatment	Post-treatment	Decrease percentage %	Pre-treatment	Post-treatment	Reduction percentage %
C1, Red betel leaf extract [ns]	1.7±0.30	0.67±0.58	60.1	13.77±7.7	9.3±7.80	32.5
C2, Moringa leaf extract [ns]	1.0 ±0.0	0.33±0.33	67.0	0.37±0.27	0.17±0.12	54.1
C3, Garlic extract [ns]	1.7±0.6	0.7±0,58	58.8	0.17±0.20	0.05±0.03	70.6

[ns] non-significant

Table 2: Pregnancy rates of Aceh cows experiencing endometritis pre-and post-treatment with red betel leaf extract, moringa leaf and garlic extracts

	Treatments		
Parameters	C1, Red betel leaf extract	C2, Moringa leaf extract	C3, Garlic extract
Number of samples	3	3	3
Number of cows cured (%)	1 (33,3)	2 (66,6)	0 (0)
Number of estrus/AI cows (%)	3 (100)	3 (100)	3 (100)
Number of pregnant cows (%)	2 (66.6)	2 (66.6)	0 (0)

Of the nine cows with endometritis, the recovery rates in C1, C2, and C3 were 33.3, 66.6, and 0.0%, respectively. However, all cows managed to show estrus symptoms after estrus induction with PGF2α. All cows that were categorized as cured (WST score 0) managed to become pregnant after being inseminated, although there were also cows with a WST score of 1 that were successfully pregnant in group C1 (Table 2).

Discussion

The reductions in the WST scores at C1, C2, and C3 were 60.1, 67.0 and 58.8%, respectively. The decrease in WST score post-treatment was followed by a decrease in total bacterial colonies ($p>0.05$). In group C1, out of 3 cows with endometritis, two cows had endometritis level 2 and one cow had endometritis level 1. Post-treatment using red betel leaf extract, the endometritis level decreased from 2-1 and 1-0 (cured). In C2 cows, all cows with endometritis had endometritis level 1. Post-treatment with moringa leaf extract, two samples decreased from 1-0 (cured), whereas in one sample there was no change in the score. In C3 cows, two out of 3 cows had level 2 and one had endometritis level 1. Post-treatment with garlic extract, two samples decreased from 2-1, but there was no change in the last sample.

The decrease in the endometritis and the total bacterial count indicated that all the extracts used in this study had antibacterial activity against *E. coli.* The antibacterial activity of red betel leaf has been reported against *S. aureus* (Bustanussalam *et al*., 2015), as well as *S. aureus* and *E. coli* (Retnaningsih *et al*., 2018; Puspita *et al*., 2018). Amanda *et al*. (2019) also reported that the aqueous extract of betel leaf could inhibit the growth of *Streptococcus pyogenes*.

The reduction rate in the endometritis and the total bacterial counts was not optimal (below 80%) which was assumed due to the type of bacteria. Generally, bacteria that infect the Aceh cows' uterus are non-specific bacteria such as *E. coli*, *Streptococcus*, *Staphylococcus*, and *Corynebacterium pyogene* and specific bacteria such as *Vibrio foetus*, *Trichomonas foetus*, and *Brucella* sp. (Rafika *et al*., 2020). Armansyah *et al*. (2022) reported that 40% of red betel leaf extract had a bactericidal action on *S. aureus*, but was not effective on *E. coli*.

Purnasari (2013) reported that moringa leaf extract was effective against one type of bacteria at a certain concentration. Moringa leaf extract (100 mg/mL) started to actively inhibit *S. aureus* with a diameter of 6.80 mm; however, a concentration of 50 mg/mL could inhibit *E. coli* with an inhibition zone diameter of 9.20 mm. Fitriah *et al*. (2017) stated that ethanolic extract of johar leaves with similar concentrations had different inhibitory effects on different bacteria. Inhibition on *S. aureus*, *E. coli*, *Micrococcus luteus* and *Shigella dysentriae* were 14.9, 12.9, 12.0 and 7.2 mm, respectively. Aceh cows used in this study were possibly mixed infected, reducing the effectiveness of moringa leaf extract in decreasing endometritis. Rafika *et al*. (2020) found that Aceh cattle diagnosed with repeat breeding had been infected by both Gram-negative bacteria (*E. coli*, *Enterobacter* sp., *Pseudomonas* sp., *Klebsiella* sp.) and Gram-positive bacteria.

Although not significant, red betel leaf, moringa leaf, and garlic extracts can reduce the level of endometritis and the total bacterial counts of Aceh cows with endometritis. This antibacterial ability was

due to the active compound in the extracts. The red betel leaf in the ethanol fraction contains flavonoids, steroids, and phenolics (Armansyah *et al*., 2022). This extract also contains chlorophyll and anthocyanin color pigments. Anthocyanin pigments are derivatives of flavonoid compounds that are widely reported to have multi-pharmacological effects including antibacterial (Khoo *et al*., 2017).

Flavonoids in plants are phenolics that bind with sugars to form glycosides and these glycosides are more soluble in polar solvents (Prayogi and Simamora, 2020). As a polar compound, flavanoid is easier to infiltrate the polar peptidoglycan layer in *E. coli*, the main bacteria causing endometritis in Aceh cattle. These compounds function as antimicrobials that poison the protoplasm, and damage cell walls, lysosomes, and microsomes due to their interaction with bacterial DNA (Sipayung, 2014; Syahrinastiti *et al*., 2015). Moringa leaves contain a short polypeptide known as 4-(alpha-L-rhamnosyloxy benzyl isothiocyanate). This compound acts directly on microbes and inhibits the microbes' growth by interfering with the cell membrane synthesis. The inhibition mechanism is similar to that of the beta-lactam group and cephalosporins or inhibition of essential enzymes (Bukar *et al*., 2010).

This suboptimal result may also be related to the low concentration of the extract used or the type of bacteria that causes endometritis in cattle. According to Nazzaro *et al*. (2013), antibacterials affect bacterial energy production at low concentrations and denature bacterial proteins at high concentrations. It was reported that the higher the moringa leaf extract used, the greater its ability to inhibit the growth of *E. coli* (Sudarwati and Sumarni, 2016). Similarly, Dima *et al*., (2016) observed that moringa leaf extract at a concentration of 20% could inhibit the growth of *E.coli* with the inhibition zone formed was 15.83 mm, however, the diameter was increased to 22.66 mm at a concentration of 80%. This indicated that the higher the concentration, the higher the active substance contained in it, causing greater inhibition activity (Wulandari *et al*., 2015).

The results of the cure rate in cows treated with moringa leaf extract were higher than those of Sutriana *et al*. (2021) who used Lugol solution with a cure rate of 33.3%. This may not be related to the decrease in the bacterial number due to the administration of the extract. This can be proven by the cure rate of 0% in the administration of garlic extract although the total bacterial count reduction was relatively high. Of the three cows inseminated in C1, C2, and C3, the pregnancy rates were 44.4, 66.6, and 0%, respectively. Bhardwaz *et al*. (2018) reported that intrauterine administration of Lugol solution of 0.3% in endometritis cows resulted in increasing the pregnancy rate from 0% (control group) to 30.0 % (treatment group). Similar results were obtained by Pandey *et al*. (2011) who reported a higher number of pregnant dairy cows in the first insemination post-treatment using 20 mL of 0.25% lugol on day 5 and 17th day of the estrus cycle.

Overall, this study indicated that the administration of red betel leaf and moringa leaf extracts can improve the reproductive performance of Aceh cattle, similar to the therapy using Lugol solution as previously reported by Ahlawat and Derashri (2010); Pandey *et al*. (2011); Ahmed and Elsheikh (2014); Alyasiri *et al*. (2015); Singh *et al*. (2017); Bhardwaz *et al*. (2018). This increase in performance may be caused by a decrease in the bacterial count in the reproductive tract of Aceh cattle, which rescues the embryonic implantation.

Conclusion

Red betel leaf and moringa leaf can enhance the reproductive performance of Aceh cows by reducing the level of endometritis and total bacterial counts and increasing the pregnancy rates of Aceh cows. Further research is needed to confirm the positive effect of these plant extracts using the higher dosage with the variation of administration duration.

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Author's Contributions

Teuku Armansyah: Designed the research participated in all experiments and drafted written.

Tongku Nizwan Siregar: Organized and designed the research and was responsible for manuscript revision.

Amalia Sutriana and Suhartono Suhartono: Participated in design research and manuscript revision.

Budianto Panjaitan: Responsible during artificial insemination, pregnancy, detection and research data analysis.

Rania Evita Agustine, Nuriani Saputri and Uti Nurlita: Conducted research.

Ethics

This is an original article that has not been published elsewhere. The corresponding author ensured that all the authors had read and approved this article's content and

that there were no conflict of interest issues involved. This study has been approved by the Ethics Committee of the Faculty of Veterinary Medicine, Universitas Syiah Kuala (Ref: 222/KEPH/IX/2021).

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