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The Effect of Red Betel Leaf (*Piper crocatum*) and Moringa Leaf Extracts on Endometritis Levels in Aceh Cows

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ABSTRACT

This study aims to determine the ability of red betel leaf (*Piper crocatum*) or Moringa leaf extracts to reduce the endometritis level in Aceh cattle. In this study, six Aceh cows aged 3-5 years, weighing 150-250 kg from the Experimental Animal Technical Implementation Unit of Faculty of Veterinary Medicine of Syiah Kuala University were used. The cows were divided into two treatment groups, namely cows with endometritis that were given red betel leaf extract (T1) and cows with endometritis that were given Moringa leaf extract (T2). Examination of the endometritis levels was carried out before and after treatment using the White Side Test (WST) method. The collection of estrus cervical mucus was needed for the WST examination, and heat induction was performed with prostaglandin F2 alpha (PGF2α) at a dose of 25 μg. Collection of cervical mucus was performed 8-12 hours after the initiation of heat. All cows with endometritis were given intrauterine extracts of red betel leaves or Moringa leaves at a concentration of 20% every 24 hours for a week at a solution volume of 20 ml. The data were then analyzed using a paired t test. The mean endometritis levels before and after treatment on K1 vs. K2 were 3.0 and 1.7 vs. 2.7 and 2.7, respectively ($P<0.05$). It was concluded that red betel leaf extract at a concentration of 20% was more effective in reducing the endometritis level of Aceh cows than Moringa leaf extract.
Keywords: endometritis; red betel leaf extract; Aceh cows

INTRODUCTION

Endometritis is defined as mild inflammation of the endometrium that usually occurs without generalized or systemic disorder after childbirth, coitus, or artificial insemination in cattle (Hussain and Daniel, 1991; Maurya *et al*., 1992). The severity of endometritis depends on the malignancy of the infecting bacteria, the number of bacteria, and the resistance of the host body. The forms of infertility that occur include early embryo death due to the influence of microorganisms or disruption of the embryo attachment to the uterine wall (Melia *et al*., 2014). Microorganisms that often enter through the cervix and vagina include *Streptococcus*, *Staphylococcus*, *Trichomonas*, *Vibrio foetus* and *coli*. The *coli* germs come from feces which may enter during careless insemination or birth assistance (Waluyo, 2014). In addition, Sari *et al*. (2016) reported that the bacteria that cause endometritis are *Streptococcus* sp., *Staphilococcus* sp., *Klebsiela* sp., *Escherichia coli*, *Bacillus* sp., and *Alkagenes fecalis*. Endometritis can also be caused by continued retention of the placenta, dystocia, uterine prolapse, and poor environment.

The incidence of endometritis varies from 34-43% in cattle (Martinez and Thibier, 1984; Heuwieser *et al*., 2000) and from 18.0-22.0% in buffalos (Sreemannarayana and Rao, 1997). Endometritis is generally characterized by the absence of estrus, white or creamy yellow discharge from the vagina, and a large uterus that cannot peel off. Subclinical endometritis is also prevalent in dairy cattle and has a negative impact on reproductive performance (Hammon *et al*., 2006). Various therapies have been carried out on cows with endometritis, such as the administration of antibiotics, prostaglandins (Drillich, 2006; Mido *et al*., 2016; Szenci, 2016), and lugol (Ahmed *et al*., 2014; Ahmed and Elsheikh 2014; Alyasiri *et al*., 2015). Giving 1% lugol can treat reproductive disorders in bovine uterine infections (Ahmed and Elsheikh, 2013). Melia *et al*. (2020) reported that an administration of 2% lugol cannot treat endometritis in Aceh cattle. Although antibiotics can treat endometritis, the use of medicinal plants as antimicrobials and immunomodulators is becoming popular because antibiotic use has side effects and toxicity (Bhardwaz *et al*., 2018). Treatment of endometritis in animal models using phytotherapy has been reported by several researchers (Dwivedi and Tripathi, 2014; Sharma *et al*., 2018; Ding and Lian, 2015; Wardani *et al*., 2017; Hafsari and Pujiastutik, 2018; Lestari *et al*., 2018; Bina *et al*., 2019). Extracts from various plants have antibacterial activity which they play a role in controlling infection (Dwidjoseputro, 1994

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cited by Dima *et al*., 2016). The use of herbal antiseptics such as red betel leaf (*Piper crocatum*) and Moringa leaves (*Moringa oleifera* Lamk) is expected to become a safer alternative to commercial antiseptics. Red betel leaf extract can play a role as gram-positive and gram-negative antibacterial (Syahida *et al*., 2013). Red betel leaves are known to contain essential oils, alkaloids, saponins, tannins, and flavonoids which function as antibacterial compounds (Putra *et al*., 2017). Moringa leaves contain high flavonoids, with quercetin being the most dominant. Andjani *et al*. (2016) reported that quercetin functions as an anti-inflammatory agent that can inhibit *Nuclear factor kappa B* (NfkB), a marker of an inflammatory reaction in endometritis.

METHODOLOGY

In this study, six Aceh cows with endometritis aged 3-5 years old, with a body weight of 150-250 kg were used. To ensure that the cows have endometritis, a White Side Test (WST) was performed. The intrauterine extract treatment was carried out every 24 hours for a week (Bhardzwad et al., 2018) with a solution volume of 20 ml (Melia *et al*., 2020). The extract concentration given was 20% (Putra *et al*., 2017).

Research Procedure

Making extracts of red betel leaves and Moringa leaves

Betel leaves are obtained from Rantauprapat (Sumatera Utara) while moringa leaves are obtained from Aceh Besar district. Leaf criteria used intact and medium-aged leaves. Red betel leaves or Moringa leaves were extracted using the maceration method with 96% ethanol as the solvent. Red betel leaves or Moringa leaves were grinded into powder, put into an Erlenmeyer flask, and 96% ethanol was added, which was then stirred for 2-3 hours. Next, it was covered with aluminum foil. After filtering, the filtrate was collected, and the solution was then evaporated to separate the extract from the solvent using a rotary evaporator until the extract of red betel leaves or Moringa leaves was free from ethanol (Lestari *et al*., 2018). Red betel leaf extract powder and moringa leaves were weighed as much as 100 g, then extracted by maceration method using 1 L of ethanol as a solvent. The maceration results obtained were then evaporated using a rotary evaporator at 50 C, 100 rpm.

Estrus Induction with PGF2 Alfa

All cows were estrous synchronized using the PGF2α hormone (Sincrovall® Mevet. MPA Veterinary Medicines and Additives-Spain) which was injected intramuscularly at a dose of 25 µg with a double injection pattern twice with 11 days interval. Heat detection was carried out in the morning (8:00 AM) and evening (16:00 PM) for 30 minutes. Cows were considered to be in heat and ready to mate when the signs, such as a red and swollen vulva and a transparent cervical mucus, appeared.

Mucus Collection

Collection of cervical mucus was done 8-12 hours after the initiation of heat (Bhat *et al*., 2014). The external genitals of female animals were cleaned using 70% alcohol and then dried with cotton. Cervical mucus was taken for endometritis diagnosis by WST test. The mucus samples were collected by aspiration method using a sterile 50 ml pipette connected to a catheter tube. A sterile dropper was inserted into the vagina followed by rectal palpation to ease guide the dropper into the cervix or vaginal folds.

Diagnosis of Endometritis with WST

The WST test was carried out twice, namely before and after medical therapy with red betel leaf extract. A total of 1 ml of cervical mucus was heated with 1 ml of 5-10% sodium hydroxide to boiling point. The solution was then cooled and the intensity of the color change was observed. In normal cattle without infection, the solution would be cloudy or have no color (score 0); in cattle with a mild infection, the solution would be light yellow (score 1); in cattle with moderate infection, the solution would be yellow (score 2); and in cows with severe infection, the solution was dark yellow (score 3) (modified Bhat *et al*., 2014). The data obtained were analyzed using the t test.

RESULT AND DISCUSSION

WST test was performed on cervical mucus obtained from three samples of cows before and after treatment, which is reported in Table I. The WST test was used to see any changes in the color of cervical mucus from cloudy to yellow. In this test, cervical mucus was collected aseptically from infected cattle and heated with 5% sodium hydroxide (Mandhwani *et al*., 2017). The color change is considered positive if the color turns yellow as an indicator of infection. The intensity of the color reaction depends on the number of leukocytes present in the uterine mucus. Cervical mucus in subclinical and clinical endometritis increases the number of leukocytes to normal (Bedewy and Rahawy, 2019). Normal cervical mucus has a lower number of leukocytes so that it does not cause discoloration, whereas, in clinical or subclinical endometritis, cervical mucus

Table I. White Side Test results for cervical mucus estrus in cattle with endometritis before and after treatment with red betel leaf or Moringa leaf extract

Color intensity	Infection score	The number of samples in the red betel leaf group		The number of samples in the Moringa leaf group	
		Before	After	Before	After
No color	0 (normal)	0	0	0	0
Light yellow	1 (mild)	0	1	0	0
Yellow	2 (moderate)	0	2	1	1
Dark yellow	3 (severe)	3	0	2	2
Average		3.0[a]	1.7[b]	2.7[a]	2.7[a]

[a, b] Different superscripts on the same line show significant differences (P <0.05)

contains an increased number of leukocytes that cause a color reaction. The color reaction is due to the ribonucleic acid in the nucleus of white blood cells that reacts with 5% NaOH and produces a yellow color (Gupta *et al*., 2011).

The three samples showed a decrease in the level of endometritis after red betel leaf extract administration therapy. The effectiveness of the red betel leaf extract in reducing the level of endometritis is probably due to the accuracy of the dosage and its ability to function as an antibacterial agent against the types of bacteria that cause endometritis in Aceh cows.

The non-specific bacteria that commonly infect the uterus of Aceh cows are *Escherichia coli*, *Staphylococcus*, *Streptococcus*, and *Corynebacterium pyogenes*, while the specific bacteria include *Brucella* sp., *Vibrio foetus,* and *Trichomonas foetus* (Rafika *et al*., 2020). Several studies have reported the antibacterial ability of red betel leaf against skin infections caused by *S. aureus* (Retnaningsih *et al*., 2018) and gram-positive *S. aureus* and gram-negative bacteria *E. coli* (Puspita *et al.*, 2018; Januarti *et al*., 2019). Red betel leaf extract can inhibit and kill *S. aureus* at a concentration of 25% and *E. coli* at a concentration of 6.25% (Pratiwi and Irma, 2012). Betel leaf extract has an inhibition zone against the growth of *S. aureus* bacteria, with the highest inhibition zone shown at a concentration of 20% of 1.64 mm (Bustanussalam *et al*., 2015). Amanda *et al*. (2019) reported that betel leaf boiled water has antibacterial ability against *Streptococcus pyogenes* bacteria. However, no reports of the antibacterial ability of red betel leaf have been found against other types of bacteria that cause endometritis in Aceh cattle.

The antibacterial ability of red betel leaves is due to the content of antibacterial compounds in red betel leaves such as tannins, alkaloids, saponins, and flavonoids. These compounds can inhibit growth for several types of bacteria (Reveny, 2011). Alkaloids have antibacterial properties. The suspected mechanism works by disrupting the peptidoglycan constituent components in bacterial cells so that the cell wall layer is not formed completely and causes cell death (Pratiwi and Irma, 2012). Tannins have antibacterial activity because they can damage bacterial cell membranes. Astringent tannins can induce the formation of complexes of bonding compounds to enzymes or microbial substrates and the formation of a complex of tannin bonds to metal ions which can increase the toxicity of the tannins themselves. The antibacterial effects of tannins include reactions with cell membranes, enzyme inactivation, and destruction or inactivation of the function of genetic material (Syafriana and Rabitha, 2017). Saponins have different pathways in inhibiting bacterial growth, namely by disturbing the stability of the cytoplasm so that the cytoplasm leaks and results in cell death (Vifta *et al*., 2017).

Red betel leaf extract contains chlorophyll and anthocyanin color pigments. In this study, medium-aged red betel leaves were used so that the anthocyanin pigment content increased and the chlorophyll content decreased. Essential oils consist of hydroxy chavicol, betlephenol, chavicol, cavibetol, estragole, eugenol, metileugenol, cineole, and carvacrol, all of which can damage bacterial cell walls. The carvacrol, eugenol, and chavicol groups have a mechanism of action by destroying the cytoplasmic membrane and denaturing proteins and can prevent the formation of bacterial cell walls (Hasanah *et al*., 2020).

The components of the essential oils of red betel leaves are: a-thuyene, a-pinene, sabinene, ß-myrcene, terpinene, ß-phellandrene, γ-terpinene, ß-terpineol, terpinolene, a-terpineol, copaene, caryophyllene, caryophyllene, and germacrene D. Essential oil components such as a-pinene, a-terpinene, and γ-terpinene have an antimicrobial activity that damages cell

membranes or inhibits the formation of cell walls in fungi. These components destroy the cell membrane by binding or inhibiting the formation of ergosterol so that the cell membrane will be damaged or leak and eventually be destroyed (Rachmatiah *et al*., 2018).

The main components contained in essential oils are phenols and their derivative compounds. The other components of red betel leaves are 3.1% protein, 6.9% carbohydrates, 2.3% minerals, 2% tannins, 1,8-cineol terpenoids, cadinene, camphene, caryophyllene, limonene, pinene) (Rizkita *et al*., 2017). The antibacterial power of essential oils is due to the presence of phenolic compounds and their derivatives. Flavonoids and tannins are phenol derivatives that can cause denaturation and coagulation of bacterial cell proteins (Moerfiah dan Fira, 2011). Generally, flavonoid compounds in plants are phenolic compounds that bind to sugar to form glycosides that are more soluble in polar solvents (Prayoga *et al*., 2019).

Phenol is a toxic compound that causes the three-dimensional structure of the protein to be disrupted and opens into a random structure without any damage to the covalent skeleton structure. This causes the protein to change its nature; its biological activity is damaged so that the protein cannot perform its function. As a result of denatured protein in bacterial cells, all metabolic activities are catalyzed by enzymes so that microbes or fungi cannot live (Utami *et al*., 2015).

The mechanism of action of phenol as an antibacterial agent is its ability to function as a toxin in the protoplasm that damages, penetrates the cell wall and precipitates bacterial cell proteins (Syahrinastiti *et al*., 2015). The process of making red betel leaf extract was carried out by the maceration method. Maceration was chosen because through this method the active compounds could be extracted well through immersion without heating so it did not damage the components of the compounds, which could not withstand high temperatures. Immersion was done so that the solvent could penetrate the cell wall and enter the cell containing the active substance and the active substance contained in the cell would dissolve in the solvent (Hidayah *et al*., 2013).

The ethanol solvent is polar so this solvent is often used to identify bioactive compounds. The difference in ethanol concentration can change the polarity of the solvent so that it affects the solubility of bioactive compounds. The concentration of ethanol affects the bioactive components; the higher the ethanol concentration, the higher the bioactive components produced. The higher the ethanol concentration, the higher the yield of the extract produced, but the polyphenol content reaches an optimum at certain ethanol concentrations (Yasa *et al*., 2019).

The effectiveness of extraction has the principle of *like dissolves like*, where a compound will dissolve in a solvent with the same polarity. The solvent used in this study was 96% ethanol, which is a polar solvent. Apart from ethanol, other polar solvents are methanol, acetone, and water (Verdiana *et al*., 2018). The factors that affect the extraction process are the type of extraction, the ratio of solvent, time, temperature, and the amount of solvent used (Rifai *et al*., 2018). The antimicrobial power of the ethanol extract of red betel leaf has stronger antimicrobial activity than the ethanol fraction and n-hexane fraction, while the water fraction is ineffective (Achwandi *et al*., 2015).

The average WST results before and after treatment of Moringa leaf extract were 2.7 each, as presented in Table I. Treatment or therapy of endometritis with Moringa leaf extract with a concentration of 20% in this study did not show effectiveness because it did not produce significant changes or actual decreased levels of endometritis. According to Nazzaro *et al*. (2013), the ability of antibacterial compounds at low concentrations affects the energy production of bacteria, while at high concentrations it can denature the protein. Sudarwati and Sumarni (2016) reported that the higher the Moringa leaf extract used, the greater the inhibition zone formed, therefore the greater its ability to inhibit the growth of *E. coli* bacteria. At a concentration of 20% the inhibition zone is only 15.83 mm but increases to 22.66 mm at a concentration of 80% (Dima *et al*., 2016).

According to Brooks *et al*. (2005), the difference in the diameter of the inhibition zone at each concentration is due to the difference in the size of the active substance contained in that concentration. The greater the concentration, the greater the active substance components contained in it, so the inhibition zone formed is also bigger. The increase in the inhibition zone along with the increase in the concentration of Moringa leaf extract suggests that even higher concentrations are possible to produce a higher inhibition zone.

Another factor that influenced the failure of Moringa leaf extract to reduce the level of endometritis in this study may be the type of bacteria that infects the cow. Fitriah *et al*. (2017) reported that ethanol extract of *johar* leaves; at the same concentration it had different inhibition for each type of bacteria. Inhibition against *Staphylococcus aureus* bacteria, *E. coli*, *Micrococcus*

luteus, and *Shigella dysentriae* were 14.9 mm, 12.9 mm, 12.0 mm, and 7.2 mm, respectively. It is suspected that the cows in this study were infected by several types of bacteria and were not having a single infection, causing Moringa leaf extract at a concentration of 20% unable to reduce the level of endometritis. Rafika *et al*. (2020) reported that Aceh cows that experienced repeat breeding were infected by several gram-negative bacteria, namely *E. coli, Pseudomonas* sp., *Enterobacter* sp., and *Klebsiella* sp., and still had a chance of being infected with gram-positive bacteria.

The difference in the sensitivity of bacteria to the antibacterial substances in Moringa leaf extract is due to the influence of the bacterial cell wall structure; Gram-positive bacteria have a simpler cell wall structure while gram-negative bacteria have a much more complex cell wall structure. The cell wall of gram-positive bacteria has peptidoglycan and teichoic acid structure. The bioactive compounds that are thought to be present in Moringa leaf extract are flavonoids, saponins, and alkaloids, but the compounds that are thought to be the most active in the extract are flavonoids. Flavonoids are polar compounds, so it is easier for them to penetrate the polar peptidoglycan layer than nonpolar lipids such as those in E. *coli*. This compound functions as an antimicrobial agent that damages lysosomes, cell walls, and bacterial microsomes because it interacts with bacterial DNA. The short polypeptide called *4-(alpha-L-rhamnosyloxy benzyl isothiocynate)* in Moringa leaves acts directly on microbes and produces growth inhibition by interfering with cell membrane synthesis. The mechanism of action is similar to beta-lactam antibiotics and cephalosporins or essential enzyme inhibition (Suarez *et al*., 2003; and Bukar *et al*., 2010).

CONCLUSION

It was concluded that red betel leaf extract at a concentration of 20% was more effective in reducing the endometritis level of Aceh cows than Moringa leaf extract.

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