INTRODUCTION

The green betel (Piper betle Linn.) has various traditional and medicinal uses, namely a mouth freshener, cardiac tonic, antipyretic, anticarcinogenic, anti-inflammatory, immunomodulatory, and anti-thrombotic activities (Chauhan, Aishwarya, Singh, & Tiwari, 2016). Classified into the family Piperaceae, it contains nutrients, minerals, phytochemicals, vitamins, and antioxidants (Chauhan, Aishwarya, Singh, & Tiwari, 2016; Sripradha, 2014). Historically, the betel leaf was used as a natural remedy for several diseases (Padma, Lalitha, Amonkar, & Bhide, 1989; Sripradha, 2014). It is extensively cultivated in India, Sri Lanka, Malaysia, Thailand, Taiwan, and other Southeast Asian countries (Chauhan, Aishwarya, Singh, & Tiwari, 2016; Datta, Ghoshdastidar, & Singh, 2011).

In the dairy industry, one of the important diseases in cattle is mastitis (Patel, Kunjadia, Koringa, Joshi, & Kunjadiya, 2019). Mastitis is characterized by inflammation of the mammary gland caused by infection with bacterial pathogen group (Kudi, Bray, Niba, & Kalla, 2009). Staphylococcus, Enterococcus, Escherichia, and Streptococcus are the most common bacterial genera that cause mastitis (Patel, Kunjadia, Koringa, Joshi, & Kunjadiya, 2019). Kudi, Bray, Niba, & Kalla (2009) reported that mastitis can be differentiated into clinical and subclinical mastitis, and dairy cattle often acquire the subclinical condition.

In eastern China, Streptococcus agalactiae is one of the bacterial pathogens commonly found in dairy cattle with subclinical mastitis (Yang et al., 2013). This species, referred to as a group B streptococcus, is the leading cause of bovine mastitis in the dairy cattle industry worldwide (Tian et al., 2019; Yang et al., 2013). Another bacterial pathogen causing mastitis is Escherichia coli (Desloire, Valiente Moro, Chauve, & Zenner, 2006). Mastitis frequently affects high-producing cows in dairy herds and in severe cases, may cause death (Desloire, Valiente Moro, Chauve, & Zenner, 2006).

Prevention of mastitis is typically through the administration of a chemical antiseptic; however, an alternative to inhibit the growth of bacteria using a natural antibacterial compounds would be desirable. According to Dipali & Palshikar...
(2015), the essential oils found in betel leaf used at concentrations of 1%–5% inhibit the growth of bacteria such as S. agalactiae, S. epididymis, and E. coli. The antibacterial compounds produced by betel leaf are phenols and their derivatives (Shah, Garg, Jhade, & Patel, 2016; da Silva et al., 2017). The levels of essential oils, diastase, and sugar are higher in young betel leaves than in old leaves.

The green betel leaf can be used as a natural antibacterial agent; in this study, we focused on the inhibition of S. agalactiae and E. coli in dairy cattle, the two causal agents of mastitis.

**MATERIALS AND METHODS**

This research was conducted from January to February 2017 in the Laboratory of Plant Disease, Plant Pest and Disease Department, Faculty of Agriculture, Universitas Brawijaya and Laboratory of Microbiology, Faculty of Science and Technology, Islamic State University of Malang, East Java, Indonesia. The isolates of S. agalactiae and E. coli were provided and cultured by the Laboratory of Plant Disease (Bacteriology subdivision). Young (3rd leaves) and old (8th leaves) leaves of green betel were collected from Malang city. The antibacterial tests were conducted in the Laboratory of Microbiology by measuring the inhibition zone diameter of betel leaf extracts.

A completely randomized design with a nested pattern was adopted to examine six treatments with six replicates. The two physiological ages of betel leaf (young and old) were both compared at three different concentrations of crude water extract, 10%, 20%, and 30%. The procedure of preparing crude water extract of green betel (Piper betle L.) is as follows: 1) choosing and washing betel leaves (3rd leaves for young leaf and 8th leaves for old leaves), 2) draining the washed leaves, 3) cutting and weighing leaves as 10, 20, and 30 g for each treatment, 4) putting betel leaves into a beaker glass containing 100 ml of sterilized aquades, followed by aluminum foil covering, and 5) heating betel leaves at 100°C for 15 minutes.

Nutrient agar (NA) media were prepared as previously described (Cappuccino & Sherman, 2005). NA (5 g) was reconstituted with 250 ml sterilized distilled water in 500 ml glass beakers, covered with aluminum foil, sterilized by autoclaving at 121°C at 2 atm for 15 minutes, and allowed to cool. Nutrient broths were prepared as previously described (Das et al. 2011). NB (5 g) was dissolved in 600 ml sterilized distilled water in 1000-ml glass beakers, covered with aluminum foil, sterilized by autoclaving at 121°C at 2 atm for 15 minutes, and allowed to cool.

Based on the method of Prescott, Harley, & Klein (2004), the S. agalactiae and E. coli bacteria were cultured by inoculation (100 µl) onto the NA media using a micro-pipette, spreading with an L-shaped glass rod, and incubating for 24 hours at 37°C. Inhibition zone tests were performed according the method described by Tendencia (2004). An aliquot of active bacteria (100 µl) was micro-pipetted onto a petri dish, homogenized and spread using an L-shaped glass rod and then left for 45 minutes to solidify. A paper disc (6 mm diameter) was inserted into the crude water extract of betel leaves using tweezers for 45 minutes, placed into the petri dish using tweezers, and the dish was covered and incubated for 24 hours. Clear zones formed on the paper discs were measured using a caliper. The inhibition zone diameter category was determined based on average value of the clear zone diameter, according to the method described by Pan, Chen, Wu, Tang, & Zhao (2009) (Table 1).

| Inhibition Zone Diameter | Category |
|--------------------------|----------|
| 0–3 mm                   | Low      |
| 3–6 mm                   | Medium   |
| > 6 mm                   | Strong   |

During analysis, the independent variables were designated as the three concentrations (10%, 20%, and 30%) of crude water extract of young and old betel leaves, whereas the dependent variable was designated as the diameter of the bacterial inhibition zone on the surface of the culture medium. Clear zones were observed in all treatments. Data from this study were analyzed using analysis of variance with nested pattern at a = 5%. If there were differences between treatments, Duncan’s Multiple Range Test was applied. Sigmaplot 12 software (Systat Software. Inc) was used for the statistical analysis and to create graphs.

**RESULTS AND DISCUSSION**

**Phytochemical Tests**

Qualitative phytochemical tests were performed to determine the content of secondary metabolites
found in the crude water extracts of young and old green betel leaf (Table 2). Previous studies have demonstrated that crude water extracts of green betel leaf contain various phytochemicals, including steroids, diterpenes, tannins, glycosides, flavonoids cardial, saponins, phenols, alkaloids, and coumarins (Patil, Harale, Shivangekar, Kumbhar, & Desai, 2015).

**Table 2. Levels of phytochemicals in crude water extracts of green betel leaf**

| Antibacterial compound | Young leaves | Old leaves |
|------------------------|--------------|------------|
| Tannins                | ++           | ++         |
| Saponins               | +++          | ++         |
| Flavonoids             | -            | -          |
| Alkaloids              | ++           | +          |

Remarks: (-) absent; (+) low level; (++) moderate level; (+++) high level

**Physiological Age and Extract Concentration Against S. agalactiae**

The results of the measurement of the inhibition zone diameter of crude water extract of young and old betel leaves against *S. agalactiae* are presented in Fig. 1. There were no significant differences in average inhibition zone diameter by crude water extract of young or old betel leaves at any concentration ($p > 0.05$). The classification of inhibition level, based on average inhibition zone diameter of 4–5 mm, was medium (Pan, Chen, Wu, Tang, & Zhao, 2009). As the concentration of crude water extract of young green betel leaf increased, the inhibition zone diameter also increased, with the 30% concentration inducing the greatest inhibition.

These results reflect those of Brooks, Butel, & Morse (2005) who indicated that differences in the diameter of the inhibition zone for each treatment were the result of the different extract concentrations; the higher the concentration of crude water extract, the higher the content of antibacterial substances. Young leaves had higher levels of active compounds than old leaves. This result reflects those of Akiyama, Fujii, Yamasaki, Oono, & Iwatsuki (2001) who reported that in young betel leaves, flavonoid content was 3- to 4-fold higher than in other leaves. In addition, the young betel leaves contain higher levels of essential oils, diastase and sugar than old betel leaves.

![Fig. 1. Streptococcus agalactiae inhibition zone diameter of three concentrations of crude water extract of young and old betel leaves (inhibition classification is indicated above each bar)](image-url)
Physiological Age and Extract Concentration Against \textit{E. coli}

The results of the measurement of the inhibition zone diameter of crude water extract of young and old betel leaves against \textit{E. coli} are presented in Fig. 2. There were no significant differences in inhibition zone diameter by crude water extract of young or old betel leaves at any concentration ($p > 0.05$). The classification of inhibition level, based on average inhibition zone diameter of 4 mm, was medium, except for the old betel leaf at 30% concentration, which was categorized as low. These results are related to the characteristics of the cell walls of \textit{E. coli} (gram-negative bacteria) which are more complex than those of \textit{S. agalactiae} (gram-positive bacteria).

Overall, these results show that the antibacterial effect of the betel leaf extracts was clearly highest against \textit{S. agalactiae} (Fig. 1 and Fig. 2), and it has been previously reported that \textit{E. coli} tends to be resistant to the antibacterial compounds in betel leaves (Plata, Rosato, & Wegrzyn, 2009). The strongest inhibition was exerted by 30% concentration crude water extract of young betel leaves against \textit{S. agalactiae}. In contrast, the weakest inhibition was exerted by 30% concentration extract of old betel leaves against \textit{E. coli}.

CONCLUSION AND SUGGESTION

There are three conclusions in this research. Firstly, young and old betel leaves at three concentrations of crude water extract can be used as antibacterial substances to inhibit the growth of \textit{S. agalactiae} and \textit{E. coli}. Secondly, young betel leaves at 30% concentration of crude water extract is the best treatment to inhibit the growth of \textit{S. agalactiae}. Finally, young betel leaves at 20% concentration of crude water extract is the best treatment to inhibit the growth of \textit{E. coli}. It is suggested that the phytochemical content of green betel leaves based on age and physiological concentration needs further investigation.

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