Storage Duration Effect of Kelor Leaf (*Moringa oleifera*) Extracts with Methanol against Growth of *Streptococcus agalactiae* and *Escherichia coli* Caused Mastitis in Dairy Cattle

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Abstract: The research aimed to determine the storage duration effectiveness of *Moringaoleifera*leaf extract to inhibit the growth of *Streptococcus agalactiae*and *Escherichia coli* causemastitis on dairy cows. The materials were *Streptococcus agalactiae* and *Escherichia coli* from Bacteriology Laboratory Agriculture Faculty, Brawijaya University counted as 108 CFU/ml and *Moringa oleifera* leaf. This research method was an experiment Completely Randomized Design 5 treatments and 5 replications. Storage duration treatment was P0 (control), P1 (2nd day), P2 (4th day), P3 (6th day), P4 (8th day) on the same concentrations 70%. The variable measured was the diameter of the inhibition zone. The data analyzed using ANOVA followed by the Duncan test. The results showed that *Moringa oleifera* leaf extract had difference highly significant capability to inhibit the growth of *Streptococcus agalactiae* and *Escherichia coli* ($P < 0.01$). The Capability of Moringa leaf extract to maintain bacterial growth inhibition until day 2 for *Streptococcus agalactiae* and *Escherichia coli*. Maximum storage time until day 2 to maintain the effectiveness of Moringa leaf extract in inhibiting the growth of *Streptococcus agalactiae* and *Escherichia coli*.

1 INTRODUCTION

Mastitis is an inflammation of the udder gland in dairy cows. Mastitis is caused by injury to the nipple or udder tissue so that it is infected by microorganisms (Surjowardojo, et al. 2016), mastitis can also be transmitted to other livestock. Surjowardojo (2011) mastitis can reduce milk production by 4.4 - 8.3 lt /day or 28.4% - 53.5% of total production. The decrease in production is directly proportional to the level of mastitis, so the higher the rate of mastitis the greater the decrease in milk production.Manifestations of mastitis infection can be divided into two types namely clinical and sub-clinical. Supar and Ariayanti (2008) subclinical mastitis caused by pathogenic microorganisms including Staphylococcus aureus, Streptococcus agalactiae (Tuaskal, etal. 2012), Escherichia coli and Corynebacterium bovis. Hameed and Korwin-Kossakowska (2006) mastitis bacteria are dominated by Staphylococcus aureus, Streptococcus dysagalactiae, Streptococcus agalactiae and Streptococcus uberisand Coliform bacteria especially Escherichia coli (Supar and Ariayanti, 2008) and Klebsiella. It has an impact on reducing production in large numbers while the treatment of these infections is difficult to carry out to completion and requires a large cost in its operations.Surjowardojo, etc.(2016) teat dipping is a method of preventing mastitis infection by dipping the nipple in antiseptic post milking.Moringa (Moringa oleifera) is a native plant of Indonesia that can be used as medicine. Moringa oleifera leaves are a natural ingredient of antibiotics because they have active compounds, including flavonoids (Widjawati, et al.) saponins, tannins, alkaloids, and terpenoids (Yudistira, et al. 2013). Moyo (2012) Moringa oleifera leaves have antimicrobial activity against some Gram-negative bacteria including Escherichia coli. Moringa oleifera leaves contain secondary metabolites such as essential oils, polyphenols, and saponins which have potential as antibacterial and antifungal. Fuglie (2001) added that the saponin content is 5%, tannins are 1.4% and triterpenoids are 5%. Tannins, polyphenols, and saponins have been known to damage bacterial cells by inhibiting

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protein synthesis and damaging cell membranes.Moringa oleifera leaf extract has potential as an antimicrobial the bacteria Streptococcus agalactiae and Escherichia coli, it is necessary to investigate the effect of the storage time of *Moringa oleifera* leaves extract with methanol as a solvent.on the growth of *Streptococcus agalaciae* and *Escherichia coli* bacteria causing mastitis in dairy cows.2 Manuscript Preparation
We strongly encourage authors to use this document for the preparation of the camera-ready. Please follow the instructions closely in order to make the volume look as uniform as possible (Moore and Lopes, 1999).

2 MATERIAL AND METHODS

2.1 Material

The materials were *Streptococcus agalactiae* and *Escherichia coli* which were produced by the Bacteriology Laboratory majoring in Plant Pests and Diseases, Faculty of Agriculture, Brawijaya University. The instruments were *Moring aoleifera* leaf extract, analytical scales (acc.0.1 mg), oven, grinder, 1-liter Erlenmeyer, measuring cup, rotary evaporator, funnel Buchner, vacuum pump, shaker incubator, filter paper. The instruments of bacterial inhibition tests were Petri dishes, test tubes, Spertus / Bunsen lamps, autoclaves, incubators, Erlenmeyer flasks, 500 mL measuring cups, micropipettes, tweezers, calipers, stirrers, magnetic stirrers, label paper, tissue, plastic wrap, L glass sticks, aluminum foil, Cork borer. *Moringa oleifera* leaf extract with 96% methanol solvent *Moringa oleifera* leaves are obtained from Mr. Juma'il's garden in Panarukan, Kepanjen, Malang. Mac Conkey Media Agar, MRSA media, 96% p.a methanol, 70% alcohol, and *Moringa oleifera* leave powder.

2.2 Method

This research method was completely randomized design (CRD) with 5 treatments and 5 replications. The treatment used is the storage time of Moringaoleifera leaves methanol extract at a concentration of 70%. With the following conditions:P0 = Storage day 0, P1= Storage day 2, P2= Storage day 4, P3= Storage day 6, P4= Storage day 8.

2.3 Procedure

1. Making Simplisia *Moringa oleifera* Leaves
2. *Moringa oleifera* leaves Extraction
3. Making 70% *Moringa oleifera* leaves Extract Solution Concentration according to Manu (2013).
4. Making Mac Conkay Agar (MCA) Media anonymously (2011).
5. Making Media de Mann Rogosa Sharpe Agar (MRSA) according to Anonymously (2011).
6. Inhibitory testing accordingly (Kasogi et al, 2014):

Warbung, et al (2014) the formula for calculating the inhibition zone is as follows:

$$\frac{d1 + d2}{2} - X$$

Note:
d1 = vertical diameter of the clear zone on the media.

d2 = horizontal diameter of the clear zone on the media.X = well hole (5 mm).

Susanto, etal. (2012) inhibitory zones can be categorized as follows, for diameters> 20 mm are categorized as highly strong, 11-20 mm are categorized as strong, 6-10 mm are categorized as moderate and <5 mm are categorized as weak.

2.4 Variable

The variables were diameter of inhibitory zone in the form of clear area on the surface of the medium between the extract of Moringaoleifera leaves and Streptococcus agalactiae and Escherichia colibacteria.

2.5 Data Analysis

The research method used was a completely randomized design experimental method with 5 treatments and 5 replications. The results of the data obtained were processed using ANOVA followed by Duncan's Multiple Range Test is performed.

3 RESULTS AND DISCUSSION

3.1 Inhibition Zone of *Streptococcus agalactiae* Bacterial

The results of the research of the effect of the storage duration of *Moringa oleifera* leaves methanol extract on the bacteria *Streptococcus agalactiae* are shown in Table 1.

Table 1: Inhibitory zone diameter in *Streptococcus agalactiae* bacteria.

Treatment	Inhibition Zone	Categories
T0 (Control)	22.53 ± 0.42^{c}	Highly strong
T1 (Days-2)	22.24 ± 0.29^{c}	Highly strong
T2 (Days-4)	21.62 ± 0.49^{b}	Highly strong
T3 (Days-6)	20.76 ± 0.25^{a}	Highly strong
T4 (Days-8)	20.34 ± 0.58^{a}	Highly strong

Note: Different superscripts in the same column show significant between treatments (P <0.01).

Extract storage of days 0 days-2 showed that the first and second mean values were not significantly different, and were significantly different from the 4th, 6th and 8th-day treatments. Storage of methanol extract of *Moringa oleifera* leaves is recommended until the 2nd day (P1). Al-Zubaydi*et al*. (2009) that flavonoids have broad antibacterial activity because of their complex ability to extracellular and soluble proteins as well as to precipitate proteins on the cell walls of the bacterium Kiarostami et al, (2010). In addition, these phenol compounds tend to form hydrogen bonds with cell wall proteins so that they can destroy cell membranes in bacteria. The graph of the reduction in diameter of the inhibition zone of methanol extract of *Moringa oleifera* leaves is shown in Figure 1.

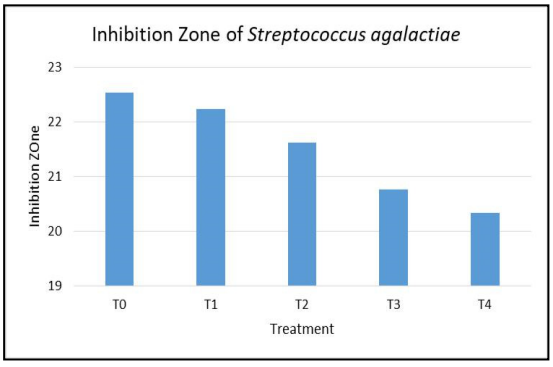

Figure 1: Inhibition zone of *Streptococcus agalactiae.*

Inhibition zone of *Streptococcus agalactiae* bacteria during storage has decreased, especially temperature factors. If the extract is stored at room temperature, the extract will quickly evaporate and cause the effectiveness of the extract to decrease bacterial growth. This is in accordance with Siswadi (2002) Decreasing the effectiveness of antimicrobial compounds is influenced by many factors including the type, age and state of microbes, concentration of antimicrobial substances, temperature and contact time, as well as the physicochemical properties of the substrate such as pH, water content and surface tension, number of components existing and other factors. Klimczak (2006) andSuhartatik*et al*. (2012) that the higher the storage temperature, the lower the flavonoid and phenolic content of the extract. The storage of plant extracts affects uterine activity and depends on extraction temperature and storage temperature.

3.2 Inhibition Zone of *Escherichia coli* Bacterial

The results of the study of the effect of the storage duration of *Moringa oleifera* leaves methanol extract on *Escherichia coli* are shown in Table 2.

Table 2:Inhibitory zone diameter in Escherichia coli bacteria.

Treatment	Inhibition Zone	Categories
T0 (Control)	22.04 ± 0.59d	Highly strong
T1 (Days-2)	21.50 ± .49cd	Highly strong
T2 (Days-4)	20.59 ± 0.74bc	Highly strong
T3 (Days-6)	19.59 ± 0.64b	Strong
T4 (Days-8)	18.13 ± 0.46a	Strong

Note: Different superscripts in the same column show significant between treatments (P <0.01).

Extract storage of days 0 days-2 shows the first and second mean values were not significantly different and were significantly different from the treatment on days 4, 6 and 8. Storage of methanol extract of *Moringa oleifera* leaves is recommended until the 2nd day (P1). The inhibitory ability of *Moringa oleifera* leaves methanol extract against Escherichia coli is weaker than that of *Streptococcus agalactiae*. The capability of methanol extract of *Moringa oleifera*leaves at a concentration of 70% is quite strong. Given the cell wall of gram-negativebacteria is more complex than the structure

of gram-positive bacteria. This is consistent with the explanation Suwito (2010), that gram-negative bacteria have a cell wall that consists of three layers. With a more complex structure of gram-negative bacteria according to Harijani (2009), making antibiotic compounds more difficult to enter the cell. In addition to the effect of shelf life, the decrease in inhibition is also due to other factors such as type, age, antimicrobial concentration, microbial state, and physicochemical properties such as pH, water content (Siswadi, 2002). A graph of the decrease in the inhibition zone diameter of methanol extract of *Moringa oleifera*leaves is shown in Figure 2.

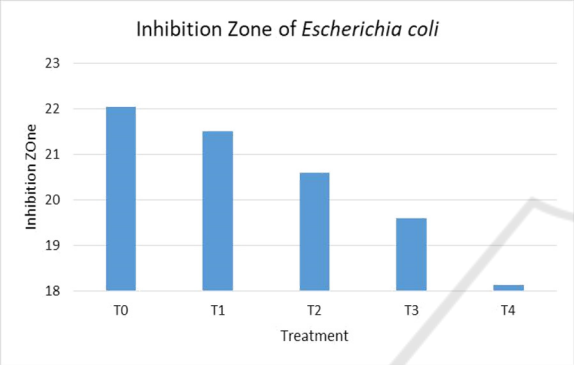

Figure 2: Inhibition zone of Eschrichia coli.

Compounds contained in Moringa leaf extracts such as flavonoids, saponins, and tannins have a function in damaging cell walls in bacteria. This is in accordance with the opinion of Khasanah, *etc*. (2014) Cell walls are the main target of being attacked by antibacterial substances contained in the Methanol extract of Moringa leaves, making it easier for tannins, saponins and flavonoids to enter the cell membrane. Cell walls are not selectively permeable so that these compounds are easily penetrated through the cell wall which will cause disruption of the integrity of the bacterial cell wall. The ability of flavonoids as an antibacterial is able to stick to bacterial cell walls and disrupt bacterial membranes, so bacteria become lysis and die. The ability of flavonoids to provide antibacterial effects includes inhibiting the function of cytoplasmic membranes, inhibiting nucleic acid synthesis, and inhibiting antibacterial activity by inhibiting energy metabolism, flavonoids inhibit oxygen consumption by interfering with the electron transport chain respiration. Permatasari, *et al* (2013) added that Saponin is included in the antibacterial group which interferes with the permeability of microbial cell membranes. Causing damage to cell membranes and causing the release of various important components from the microbial cells, namely proteins, nucleic acids, nucleotides, and others.

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