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RESEARCH ARTICLE
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Antimicrobial Content and Sensitivity Test of Citrus Peel Eco-enzyme as Natural Teat Dipping Solution in Dairy Cattle

Rizqan*, Hilda Susanty, Elly Roza, Arief, Yulia Yellita and Linda Suhartati

Department of Technology Animal Production, Faculty of Animal Science, Universitas Andalas, Padang West Sumatera 25163, Indonesia
*Corresponding author: rizqan@ansci.unand.ac.id

ABSTRACT

Post-milking teat dipping of dairy cattle into antiseptic solutions (Teat dipping) to protect the teats has been widely recognized in various dairy farming countries, which can minimize contamination of the teats from pathogenic bacteria. However, dairy farmers still rely on synthetic antiseptics, where their use can leave suitable residues indirectly in the milk dairy cows produce. The purpose of this study was to obtain the content and dose of citrus fruit peel (*Citrus reticulata*) eco-enzyme by analyzing phenol, tannin, ethanol, acetic acid and its sensitivity effect on Streptococcus sp. bacteria so that it can be used as a natural teat dipping solution in dairy cows. The method used in this research is an experimental study with a completely randomized design which is seen from the level/dose of Eco-enzyme based on citrus peel, as follows: D1. Synthetic Antiseptic (control), D2. Eco-enzyme solution (30%), D3. Eco-enzyme solution (60%), D4. Eco-enzyme solution (100%). The results of the orange peel eco-enzyme solution research show a significant effect on phenol content (11.3-75.5mgGAE/gr), tannins (0.05-0.89%1000 ppm), ethanol (1.99-2.92%), acetic acid (0.21-0.57%) and inhibition against *Streptococcus sp.* bacteria (2.87-6.75mm), and it can be concluded that citrus fruit peel (*C. reticulata*) eco-enzyme solution treatment D4 can be used as a natural antiseptic candidate for teat dipping solution in dairy cows.

Keywords: Eco-enzyme; Citrus reticulata; Teat dipping; Natural antiseptic; Dairy cows.

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INTRODUCTION

Dairy cows are livestock commonly known as milk producers that are rich in nutritional content and are needed by the community to meet animal protein needs. Good milk is not added or reduced by anything resulting from milking, either manually or using a continuous machine (Giantara et al., 2019). To get good milk free from pathogenic bacteria, it is necessary to pay attention to hygienic milking management to minimize milk contamination, a lack of hygiene can result in contamination from pathogenic microorganisms originating from the environment (Roomi et al., 2025). Contamination by pathogenic bacteria in milk begins during the milking process and continues until consumption. *Staphylococcus aureus, Escherichia coli, Salmonella sp.* and *Streptococcus sp.* are pathogenic bacteria often found in contamination (Haider et al., 2023; Chauhan et al., 2024). *Streptococcus sp.* bacteria are also the leading cause of mastitis in dairy cattle (Pribadi et al., 2020). Mastitis (udder inflammation) is an infectious inflammation, lasting acutely, subacute, or chronic, characterized by an increase in somatic cells in milk, physical changes, and milk composition, accompanied by or without pathological changes in the gland.

Hygienic milking and post-milking management are something that dairy farmers must apply. One is teat dipping, which helps protect the nipple from contamination with pathogenic bacteria by dipping or spraying anti-septic material on the nipple. The implementation of dipping in dairy cattle is widely reported in livestock development countries to minimize the occurrence of mastitis in dairy cows. The antiseptic solution used will protect the open nipple orifices after milking to reduce the contamination of pathogenic bacteria.

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However, farmers generally implement teat dipping using synthetic antiseptics such as iodine, chlorhexidine, or chlorine. According to Abni et al. (2021), synthetic antiseptic can leave residues in milk produced by dairy cows. To reduce farmers' dependence on synthetic antiseptics, environmentally friendly natural ingredients are needed as a substitute, including utilizing a citrus peel-based eco-enzyme solution as a natural teat dipping solution in dairy cattle. Researchers have widely reported eco-enzymes as environmentally friendly natural antimicrobials, such as wound medicine, disinfectants, pesticides, and insecticides (Vama & Cherekar, 2020; Riyanti et al., 2023).

The eco-enzyme solution itself is a multipurpose solution produced from anaerobic fermentation of organic materials, such as fruit/vegetable pulp, sugar, and water, with the help of ethanol-producing yeasts such as *Saccharomyces cerevisiae* and bacteria such as *Lactobacillus sp.* and *Acetobacter sp.* Fermentation in eco-enzymes is carried out for 3 months, namely, in the first month, alcohol will be formed; in the second month, vinegar will be formed so that it is slightly sour; and in the third month, enzymes will be formed. Muliarta and Darmawan (2021) stated that the acetic acid (CH3COOH) contained in eco-enzymes can kill germs, viruses, and pathogenic bacteria. In addition, Krisnawan et al. (2017) and Pande et al. (2023) reported that eco-enzyme solutions contain many antimicrobial substances, natural antioxidants, polyphenols, flavonoids, and citric acid. In order for citrus fruit peel (*Citrus reticulata*) eco-enzyme to be utilized as a natural teat dipping solution for dairy cows, the goal of this study was to determine the amount and dosage of this eco-enzyme by examining phenols, tannins, ethanol, and acetic acid as well as its sensitive impact on *Streptococcus sp.* bacteria.

MATERIALS & METHODS

Experimental Design

The citrus peel research material used is waste left over from selling unused citrus fruits on the market. The manufacture of eco-enzyme solution was carried out in the Dairy Livestock Laboratory, Faculty of Animal Husbandry, Andalas University, phenol and tannin testing in the Central Instrumentation Laboratory, Faculty of Agricultural Technology, Andalas University, ethanolic and acetic acid testing at Vahana Scientific Lab and sensitivity effect tests were carried out in the Microbiology Laboratory, Faculty of Medicine, Andalas University.

This study employed an experimental approach with four treatments and a completely randomized design (CRD): different eco-enzyme percentages (doses) and four replicates. referring to Rizqan et al. (2023) as follows:

D1. Synthetic Antiseptic (control)
D2. Eco-enzyme solution (30%)
D3. Eco-enzyme solution (60%)
D4. Eco-enzyme solution (100%)

Preparation of Eco-Enzyme Solution

Making the citrus peel-based eco-enzyme solution was carried out using a ratio of 1:3:10 with details of one part sugar, three parts peeled orange peel, and 10 parts distilled water. Mix the ingredients and put them in an airtight plastic container for anaerobic fermentation for 3 months. After three months of fermentation, the eco-enzyme solution can be harvested by filtering it first and storing it in a clean container. Fermentation can be successful when the resulting eco-enzyme solution smells slightly sour and slightly citrusy. The stages of making eco-enzyme solution can be seen in Fig. 1.

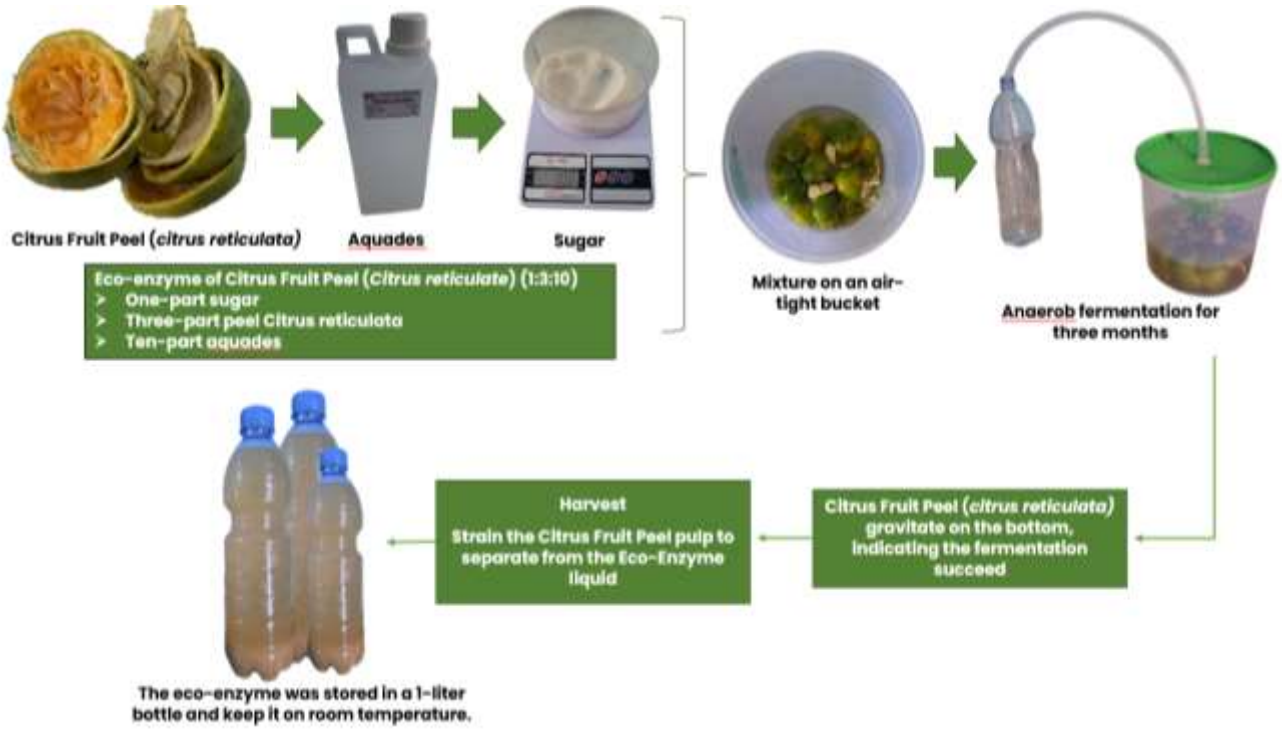

Fig. 1: Stages of making citrus peel eco-enzyme solution (Rizqan et al., 2023).

Parameters

Phenol and Tannin

Phenol and tannin testing was done using the Folin-Ciocalteu method (ISO 2005) and Fajrina et al. (2016).

Ethanol and Acetic Acid

Testing ethanol and acetic acid levels in eco-enzyme solutions was done using UV-Vis spectrophotometric methods (Kumalasari et al., 2018; Syarifuddin et al., 2022).

Sensitivity Test

The sensitivity test aims to determine the effectiveness of the eco-enzyme solution against *Streptococcus sp.* The exemplary method uses the sensitivity test (Fig. 2) by making holes in solid agar inoculated with bacteria (Kusmiyati and Agustini, 2007).

Data Analyses

The data obtained in this study were analyzed by Analysis of Variance (ANOVA), where if it shows significant results, it will be continued using the Duncan Multiple Range Test (DMRT). The applications used in data processing are Minitab 14 and OriginPro 8.5.

RESULTS & DISCUSSION

Phenols and Tannins

The results showed that phenol and tannin levels showed significant results among treatments. The highest treatment was found in the D4 treatment (Table 1 and Fig. 3); this indicates that the higher the dose of eco-enzyme, the higher the phenol and tannin levels obtained. Phenols and tannins are secondary metabolite compounds that have antibacterial activity. Phenol compounds have antibacterial properties that work by interacting with bacterial cells through an absorption process involving hydrogen bonds, this interferes with the cytoplasmic membrane's ability to function, including active transport and proton strength, according to Harborne (1987). While tannins are compounds that contain a polyhydroxy phenol core or its derivatives, such as polyphenols, which are known to have pharmacological effects, such as antibacterial, antioxidant and antiviral (Hidayah, 2016; Sunani and Hendriani, 2023), the antibacterial ability of tannins can be seen in the power to precipitate proteins, enzyme inactivation, reaction processes on cell membranes, and inhibition of the function of bacterial genetic material or destruction (Fratiwi, 2015), tannin content can also damage cell membranes in bacteria (Czerkas et al., 2024).

Table 1: Phenols and Tannins Content of Citrus Peel Eco-Enzyme Solution

Treatment	Average	
	Phenols (mgGAE/gr)	Tannins (% 1000 ppm)
D1	-	-
D2	11.3[a]	0.05[a]
D3	46.5[b]	0.69[b]
D4	75.5[c]	0.89[c]

Note: Different superscripts in the same row indicate a significant effect ($P<0.01$)

As antibacterial substances, phenol and tannin generally work by damaging the cell wall, changing the membrane's permeability, disrupting the synthesis process in proteins, and inhibiting the work of enzymes (Sunani and Hendriani, 2023). *In vitro*, tannins and phenols have antibacterial power by inhibiting a DNA topoisomerase and an enzyme, the reverse transcriptase enzyme, which causes no cell formation in bacteria; moreover, tannins can inhibit the development of bacteria by destroying their biofilm (Farha et al., 2020; Huang et al., 2024). In addition, tannin has antibacterial activity related to its mechanism that can interfere with the transport process of proteins in the layer contained in the cell, activate enzymes, and activate cell adhesins in microbes (Ngajow et al., 2013).

Fig. 2: Sensitivity Test; (a) *Streptococcus sp.* bacterial culture; (b) *Streptococcus sp.* bacterial suspension; (c) Smearing the bacterial suspension; (d) Inserting the sample into the wells.

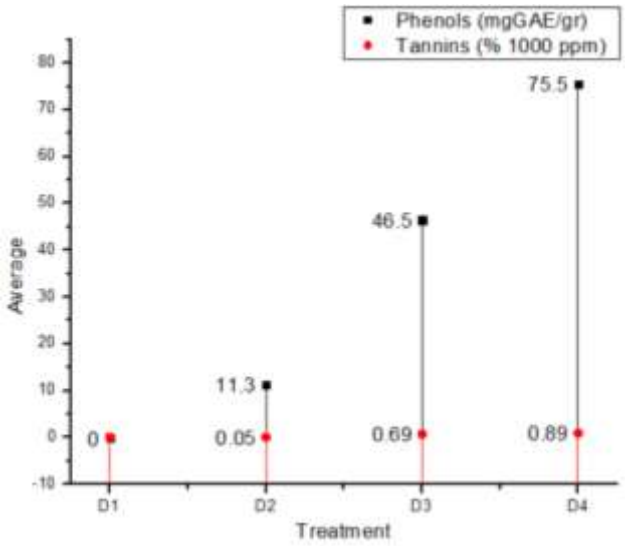

Fig. 3: Phenols and Tannins Content of Citrus Peel Eco-Enzyme Solution.

Ethanol and Acetic Acid

The results showed that ethanol and acetic acid levels showed significant results among treatments. The highest treatment was found in the D4 treatment (Table 2 and Fig. 4); this indicates that the higher the dose of eco-enzyme, the higher the ethanol and acetic acid levels obtained. Ethanol is an alcohol that is often used as a solvent, which is non-toxic and is a solvent with high polarity; because of its high polarity, ethanol can easily dissolve in water and almost all organic solvents, so it has a good ability to kill bacteria (Dianda and Suharti, 2022). Meanwhile, acetic acid is one of the organic acids that are widely used and demonstrated as antibacterial (Jamilah et al., 2008). The utilization of acetic acid has been widely reported by researchers, where washing or soaking a material using organic materials containing acetic acid can reduce the number of bacteria (Nurliana et al., 2015).

Table 2: Ethanol and Acetic Acid Content of Citrus Peel Eco-Enzyme Solution

Treatment	Average	
	Ethanol (%)	Acetic Acid (%)
D1	-	-
D2	1.99±0.009[a]	0.21±0.0001[a]
D3	2.56±0.004[b]	0.54±0.0011[b]
D4	2.92±0.052[b]	0.57±0.0031[b]

Note: Different superscripts in the same row indicate a significant effect (P<0.05).

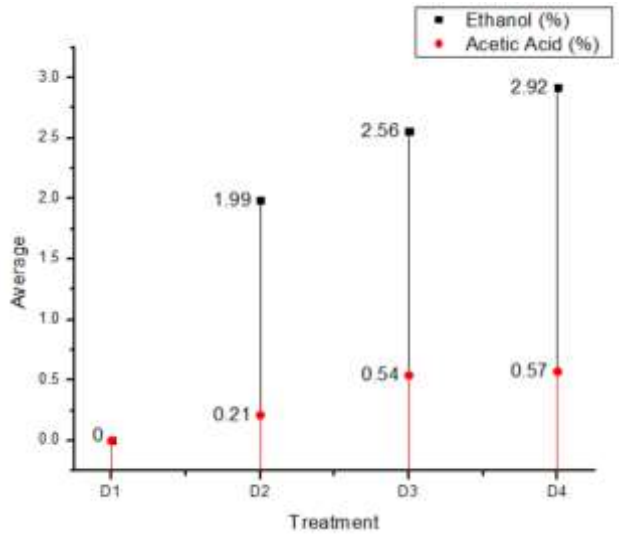

Fig. 4: Ethanol and Acetic Acid Content of Citrus Peel Eco-Enzyme Solution.

Ethanol and citric acid in the eco-enzyme solution can be used as a natural antibacterial solution for teat dipping in dairy cattle. In general, ethanol and acetic acid content in eco-enzyme solutions can inhibit bacterial growth by disrupting the acid-base balance of bacteria, causing damage and destroying bacterial cell walls (Dimariwu et al., 2020; Dianda and Suharti, 2022; Ji et al., 2023; Keyvan, 2023). Added by Dianda and Suharti (2022), Ingram (1981) stated that ethanol-containing solutions can damage the bacterial cell wall in several ways: 1) ethanol disrupts the assembly process of peptidoglycan, the main component of the bacterial cell wall, 2) ethanol can dissolve the lipid membrane of bacterial cells, which makes the cell wall more fragile, 3) ethanol also causes denaturation of proteins in the cell wall, which disrupts the function and structure of the cell wall in bacteria.

Sensitivity Test of *Streptococcus sp.* Bacteria

The results of the sensitivity test showed significant results among treatments, the best concentration of eco-enzyme solution is treatment D4 (100%), where a clear zone is formed against *Streptococcus sp.* bacteria (6.75mm) is directly proportional to the content of phenols, tannins, ethanol content, and acetic acid contained in the citrus peel eco-enzyme solution. The inhibition zone produced at a 100% (D4) eco-enzyme concentration was significantly higher than the other concentrations (Table 3 and Fig. 5). *Streptococcus sp.* is a bacterium that is often found in the udder of dairy cows and is one of the pathogenic microorganisms that can cause subclinical mastitis in dairy cows (Pribadi et al., 2020; Fesseha et al., 2021; Nikolova et al., 2022). According to. Welfalini et al. (2023) and Rahma et al. (2017) assert that an increased concentration of eco-enzyme solution results in a higher concentration of antibacterials diffusing into the agar medium, thereby producing a larger inhibition zone; conversely, a reduction in the diameter of the inhibition zone is attributed to the diminished efficacy of antibacterial compounds at lower concentrations of eco-enzyme solution. The eco-enzyme solution's phenol, tannin, ethanol, and acetic acid typically permeate into bacterial cells, where the high pH of the bacteria and the exoenzyme solution's low pH make the bacterial cytoplasm more acidic. The bacteria die as a result of the disruption of its cell activity, which damages DNA and denatures proteins. The optimum eco-enzyme solution candidate, based on the findings of the sensitivity test, was produced at a concentration level of 100%. This solution is anticipated to be used as a teat dipping solution in dairy cows and is made of readily available, natural materials that are also ecologically friendly. According to Hartanto et al. (2021), teat dipping can inhibit the entry of pathogenic bacteria through the nipple holes of cattle after milking, where the solution used will protect the nipple from microorganisms.

Table 3: Sensitivity Test of *Streptococcus sp.* Bacteria

Treatment	Average (mm)
D1	2.87±0.17[a]
D2	4.12±0.17[b]
D3	5.87±0.17[b]
D4	6.75±0.30[c]

Note: Different superscripts in the same row indicate a significant effect (P<0.05).

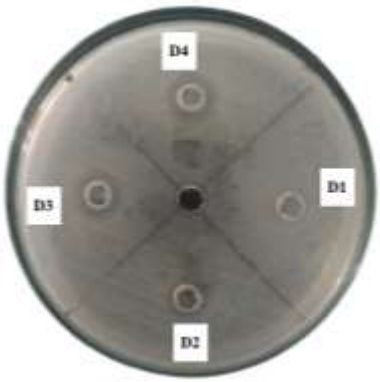

Fig. 5: Sensitivity Test of *Streptococcus sp.* Bacteria.

Conclusion

From the results of this study, it was concluded that the solution of citrus peel eco-enzymes (*C. reticulata*) contained antimicrobial substances such as phenols, tannins, ethanol, and acetic acid, which were quite good and able to inhibit *Streptococcus sp.* bacteria, which are bacteria that cause mastitis in dairy cows. Therefore, citrus peel-based eco-enzymes have good potential as a natural teat dipping solution that is environmentally friendly and readily available.

DECLARATIONS

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