

# Antibiofilm Activity of Plant Polyphenols Against *Staphylococcus Aureus* Isolated from Milk of Dairy Cows

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**Abstract**—Mastitis is a multifactorial disease, it represents a significant health, production, and economic problem in cattle farms. An important causative agent of clinical and subclinical mastitis is *Staphylococcus aureus*, whose main virulence factor is the ability to form a biofilm, which is also associated with resistance of bacteria to antibiotics. Of the 55 isolates of staphylococci that came from the milk of dairy cows with subclinical mastitis, 11 were identified as *Staphylococcus aureus*, and their ability to form a biofilm was monitored. The increase in the occurrence of resistant strains is a worldwide problem, and plant polyphenols such as quercetin and pomiferin can be used as an alternative to the use of antibiotics and inhibition of biofilm formation. The biofilm-forming reference strain *Staphylococcus aureus* CCM 4223 was used as a control. Of the 11 tested isolates, 4 were strong biofilm producers. The tested concentration of 1.56  $\mu\text{mol/ml}$  quercetin significantly reduced biofilm formation in 3 isolates. Pomiferin in the tested concentration did not inhibit biofilm formation in any of the isolates, compared to the control strain.

**Index Terms**—milk, *Staphylococcus aureus*, quercetin, pomiferin, biofilm

## I. INTRODUCTION

Mastitis is currently among the most economically serious diseases of cattle, which, in addition to the negative impact on the health of the dairy cow, results in large financial losses associated with the rejection of dairy cows, costs for treatment and disinfectants, veterinary interventions, low productivity and rejection of milk. Mastitis is a multifactorial disease, but its primary cause is an infection caused by a pathogen. Important causative agents of clinical and subclinical mastitis include *Staphylococcus aureus* [1]. *S. aureus* belongs to pyogenic bacteria, whose main virulence factor is, in addition to the production of enzymes and toxins, the ability to form a biofilm [2]. The most significant cause causing problems with mastitis eradication is the presence of biofilm in dairy cows affected by this disease [3].

Quercetin and pomiferin belong to plant polyphenols that occur naturally in most plants, especially in flowers, leaves, seeds and fruits. Plant polyphenols are secondary metabolites of plants with a distinct aroma, antiphlogistic and antibacterial effects [4]. The antibacterial action of plant polyphenols has

multiple mechanisms of action, for example, they interact with bacterial proteins, damage the cytoplasmic membrane, affect membrane permeability, inhibit nucleic acid synthesis, cell wall synthesis, and also affect energy metabolism. Some plant polyphenols also show antibiofilm activity [5].

The goal of our work was to monitor the antibiofilm activity of quercetin and pomiferin against *S. aureus* isolates that came from the milk of dairy cows with a subclinical form of mastitis.

## II. MATERIALS AND METHODS

### A. Identification of isolates

All 55 isolates of staphylococci came from milk of dairy cows with subclinical form of mastitis. Isolates were stored at  $-80\text{ }^{\circ}\text{C}$  in Microbank cryotubes (Pro-Lab, Canada). All staphylococci were identified using culture and biochemical methods. From the nutrient media, blood agar was used to evaluate the hemolytic activity of staphylococci and Baird-Parker agar was used to measure the lecithinase activity of staphylococci. Phenotypic identification of the isolates was performed using the STAPHYTest 24 biochemical series (Erba Lachema, Czech Republic). Only *Staphylococcus aureus* isolates were subjected to further testing.

### B. Testing of biofilm activity of isolates

All identified *S. aureus* isolates were tested for their ability to form a biofilm using the modified O'Toole method. A suspension with a value of 1 McFarland was prepared in a sterile physiological solution, and then 100  $\mu\text{l}$  of the thus prepared suspension was added to 100  $\mu\text{l}$  of modified BHI (37 g of BHI, 10 g of glucose and 20 g of NaCl/liter). The microtitre plates were incubated in a thermostat at  $37\text{ }^{\circ}\text{C}$  for 24 hours. After incubation, the medium was aspirated and the wells were stained with 0.1 % crystal violet solution (200  $\mu\text{l}$ ) and incubated at room temperature for 30 minutes. After washing and drying the wells, the crystal violet bound to the adhered cells was extracted with 30 % acetic acid (200  $\mu\text{l}$ ). The optical density was determined spectrophotometrically by measuring the absorbance at a wavelength of 550 nm, using a SYNERGY READER 4 device (BioTek, Merck, SRN). *Staphylococcus aureus* CCM 4223 was used as the reference biofilm-forming strain (Czech collection of microorganisms, Brno).

### C. Testing the antibiofilm activity of quercetin and pomiferin

Quercetin and pomiferin were diluted in 10 % DMSO (Honeywell) and modified BHI to a concentration of 1.56

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$\mu\text{mol/ml}$ , which was determined based on previous measurements as the minimum inhibitory concentration (MIC). A suspension of 1 McFarland density isolates (100  $\mu\text{l}$ ) was added to 100  $\mu\text{l}$  of diluted quercetin and pomiferin. The microtitre plates were incubated in a thermostat at 37 °C for 24 hours. After incubation, the antibiofilm activity of quercetin and pomiferin was tested as described above. *Staphylococcus aureus* CCM 4223 (Czech Collection of Microorganisms, Brno) was used as the reference biofilm-forming strain.

#### D. Statistical evaluation of the results

The achieved results were evaluated using the Prism statistical program using one-way ANOVA - Tukey's test.

### III. RESULTS AND DISCUSSION

All staphylococcal isolates were identified by culture, microscopy and biochemical tests. Of the 55 staphylococcal isolates, 11 were identified as *Staphylococcus aureus*, which were tested for biofilm formation ability. The results were compared with the biofilm-forming strain *Staphylococcus aureus* CCM 4223. A significantly lower biofilm-forming ability was found in 7 isolates of *S. aureus* (S8, S21, S26, S32, S71, S83, M31) compared to the control strain (Fig. 1).

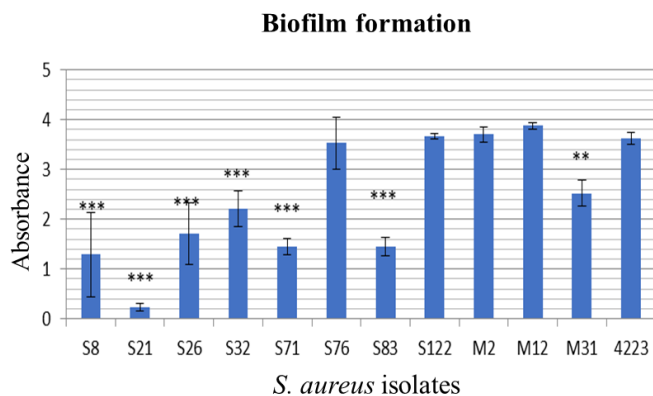


Fig. 1. Biofilm formation in staphylococcal isolates  
p<0.001\*\*\* p<0.01 \*\*

A concentration of 1.56  $\mu\text{mol/ml}$  was used to test the antibiofilm activity of quercetin and pomiferin, which was determined as MIC based on previous measurements against the control strain *Staphylococcus aureus* CCM 4223. The tested concentration of pomiferin against *S. aureus* isolates did not significantly inhibit biofilm formation compared to the control strain. Conversely, quercetin in the used concentration significantly reduced biofilm formation in 3 isolates of *S. aureus* (S8, S71, S83) compared to the control strain (Fig. 2 and Fig. 3).

#### Antibiofilm activity of pomiferin

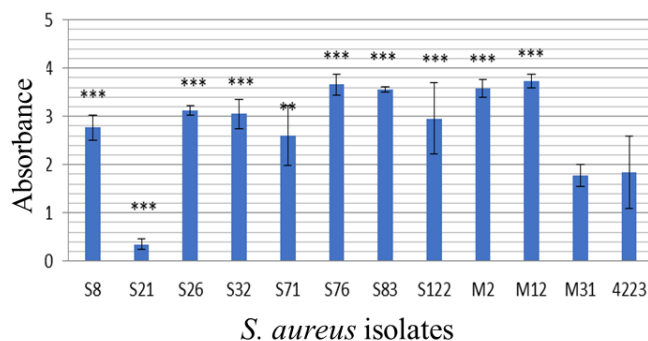


Fig. 2. Antibiofilm activity of pomiferin  
p<0.001 \*\*\* p<0.01 \*\*

#### Antibiofilm activity of quercetin

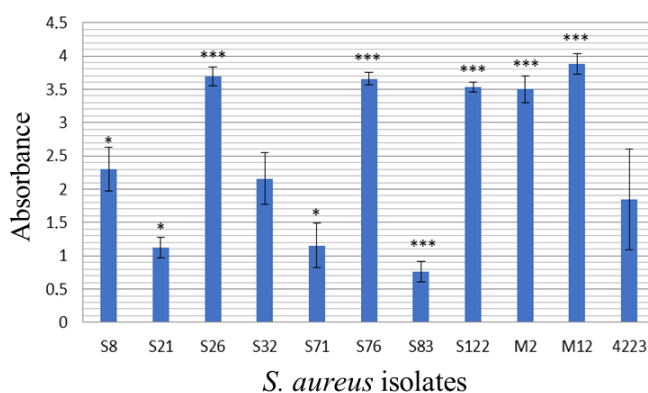


Fig. 3. Antibiofilm activity of quercetin  
p<0.001 \*\*\* p<0.05 \*\*

The prevalence of *S. aureus* as a causative agent of mastitis in dairy cows varies worldwide. As reported by Girardini et al. [6] in 2016, *S. aureus* was the causative agent of mastitis in 21.62 % of mastitis cases in Brazil. Belay, Mohammed and Wasihun [7] recorded a total of 422 cows suffering from mastitis between February 2020 and September 2020 in southern Ethiopia, of which *S. aureus* was the most isolated pathogen with a prevalence of up to 42.6 %. We are currently facing a rather complex situation where antibiotics are becoming less and less effective in the fight against *S. aureus*. As reported by Slobodníková et al. [8] the aforementioned plant polyphenols could be an alternative way in the treatment of infections caused by staphylococci. According to Kim et al. [9] quercetin shows antibiofilm activity at MIC 16-32 mg/l against *S. aureus*. As reported by Dharmaratne et al. [10] pomiferin shows strong activity against *Cryptococcus neoformans* and moderate activity against MRSA, which could prove its antimicrobial activity.

#### IV. CONCLUSION

In our work, we focused on alternative options for fighting staphylococcal infections. Staphylococci are the most common causes of mastitis in dairy cows, and the occurrence of resistant strains of staphylococci is a worldwide problem. The ability to form biofilm significantly reduces the effectiveness of antibiotics, so alternative options for inhibiting biofilm formation such as polyphenols open up new possibilities.

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