Antibacterial and antibiofilm activity of selected polyphenolic compounds: An *in vitro* study on *Staphylococcus aureus*

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Staphylococcus aureus is a human pathogen that causes serious infections with high mortality rates. The antimicrobial activities of eight phenolic compounds (caffeic acid, chlorogenic acid, ferulic acid, morin, quercetin, isoquercitrin, rutin and hesperidin) were evaluated against planktonic and biofilm forming _Staphylococcus aureus_ cells. Their ability to prevent biofilm formation via interference with bacterial cell adhesion and to reduce biomass of 24 h old biofilms has been addressed in this study. The antibacterial activities (MIC/MBC) were demonstrated using the microdilution method and the investigated compounds showed good activity against _S. aureus_ isolates, with MICs in the range 0.05-0.4 mg/mL. Also, they exhibited promising antibiofilm potential in dose depended manner. Among all tested compounds, morin and quercetin showed the best antibiofilm activity at MIC values. The observed antimicrobial potential of the studied natural products can serve as a starting point towards development of novel plant-based therapeutics for the treatment of common infections such as the ones caused by _S. aureus_.

Key words: *antibacterial activity; phenolic compounds; Staphylococcus aureus; biofilm*

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1. INTRODUCTION

Staphylococcus aureus is an opportunistic pathogen frequently linked to skin and soft-tissue infections, osteomyelitis, septic arthritis, chronic sinusitis and tonsillitis (Chalmers and Wylam, 2020; Kostić et al., 2020a). In the case of bloodstream infection caused by this bacterium there is a serious concern due to high mortality rates: in the United States among 120000 *S. aureus* bloodstream infections recorded in 2017, 20000 were with death outcome (Kourtis et al., 2019). Chronic and persistent infections by this microorganism are correlated with its ability to establish biofilms (Figueiredo et al., 2017) – trait that is directly linked to antimicrobial resistance (Manandhar et al., 2018). Resistance of biofilms is caused by lower ability of drugs to penetrate through biofilm matrix and different physiology of biofilm cell compared to the cells growing in planktonic phase (Craft et al., 2019). Biofilms can be established on indwelling medical devices; including implanted artificial heart valves, catheters and joint prosthetics and these usually lead to increased duration of patients hospital stay. The process of biofilm formation starts by attachment of cells on the surface, while next phases include accumulation/maturation and subsequent detachment and dispersal of mature biofilms (Moormeier and Bayles, 2017). The main constituents of biofilm matrix include oligosaccharides, DNA, proteins and teichoic acids while a cyclic peptide, autoinducing peptide, serves as a messenger in the intercellular bacterial communication during biofilm establishment (Craft et al., 2019). Recent studies have highlighted the potential of different agents against *S. aureus* biofilms including mushrooms (Kostić et al., 2020b), fruit peel (Stojković et al., 2018), acidic amino acids (Warraich et al., 2020) and essential oils (Kerekes et al., 2019), but the search for efficient antibiofilm therapeutics is still running.

Compounds of natural origin have been seen as an attractive source of novel anti-quorum sensing agents including the ones with antibiofilm activities (Ćirić et al., 2020). Phenolic compounds are all over distributed phytochemicals found in majority of plant organs and tissues, including vegetables and fruits. They are specialized metabolites which biosynthesis goes through the phenylpropanoid and shikimic acid pathways. These compounds possess numerous bioactivities and, although they are not nutrients, dietary intake provides health-protective effects. Besides antioxidant activity, as one of the most studied bioactivities of phenolic compounds, an-

timicrobial potential has also been explored (Smiljkovic et al., 2017; Stojković et al., 2013). However, their antibiofilm potential is in much lower proportion revealed compared to their antimicrobial effect towards planktonic cells (Smiljković et al., 2019).

Some recent studies (Ivanov et al., 2020; Slobodníková et al., 2016) highlighted a significant antibiofilm potential existing among the molecules from this group - a feature deserving more detailed experiments. This study aimed to further enlighten potential antimicrobial and antibiofilm role of selected phenolic compounds such as caffeic acid, chlorogenic acid, ferulic acid, morin, quercetin, isoquercitrin, rutin and hesperidin towards *Staphylococcus aureus* as bacterial model system for the study.

2. MATERIALS AND METHODS

2.1. Compounds collection

The following compounds were purchased from Extrasynthese (GenayCedex, France): caffeic acid, chlorogenic acid, ferulic acid, morin, quercetin, isoquercitrin, rutin and hesperidin. The selected antibiotic amoxicillin with clavulanic acid was purchased from Hemofarm (Vršac, Serbia). The phenolic compounds were dissolved in 30 % ethanol, and antibiotic was dissolved in sterile water in concentration 1 mg/mL.

2.2. Microorganisms

Two *S. aureus* strains (clinical isolate and ATCC 11632) were used. Clinical isolate was obtained from palatine tonsil of a patient after obtaining informed written consent, at Otorhinolaryngology clinic at Clinical Hospital Center Zvezdara, Belgrade, Serbia.The species were maintained in Triptone Soy Agar (TSA, Torlak, Serbia). The microorganisms are deposited at the Mycological Laboratory, Department of Plant Physiology, Institute for Biological Research "Siniša Stanković", Belgrade, Serbia.

2.3. Antibacterial susceptibility test

Microdilution method with some modifications (Kostić et al., 2017) was used to examine antibacterial activity of selected phenolic compounds. MICs were observed as the lowest concentrations with no visible bacterial growth. MBCs were determined by serial sub-cultivation of 10 μL into microtiter plates containing 100 μL of broth per well and further incubation at 37 °C for 24 h. The lowest concentration with no visible growth was defined as MBC. All experiments were repeated two times.

2.4. Inhibition of biofilm formation

The ability of phenolic compounds to inhibit biofilm formation was determined as described previously with some modifications (Kostić et al., 2020b). *S. aureus* cells were incubated in 96 well microtiter plates with adhesive bottom (Sarstedt, Germany) with MIC and sub-MIC concentrations of tested phenolic compounds at 37 °C for 24 hours. Then, wells were washed twice with sterile PBS (Phosphate buffered saline, pH 7.4), and biofilms were fixed with methanol for 10 min; next, methanol was removed and the plate was air dried and stained with 0.1 % crystal violet (Bio-Merieux, France) for 30 min. Wells were washed with water, air dried and 100 μL of 96 % ethanol (Zorka, Serbia) was added to dissolve bounded crystal violet. The absorbance was read at 570 nm using a plate reader. The percentage of inhibition of biofilm formation was calculated by the formula:

$$\text{Inhibition (\%)} = \frac{A_{control} - A_{sample}}{A_{control}} \times 100$$

2.5. Inhibition of formed biofilm

Potential of selected phenolic compounds to disturb established biofilms was determined according to Smiljković et al. (2018). Strains of *S. aureus* were grown in Triptic soy broth enriched with 2 % glucose in microtiter plates with adhesive bottom for 24 h at 37 °C. Wells were washed twice and the remaining biofilm was treated with tested compounds for 60 s at MBC values. The wells were washed; the remaining biofilm was fixed with methanol and after air drying, it was stained with 0.1 % crystal violet. After dissolving the stain in ethanol, the absorbance was read and the percentage of destruction of already formed biofilm was calculated as mentioned above.

Table 1. Minimal inhibitory (MIC) and minimal bactericidal concentrations (MBC) of phenolic compounds (mg/mL) against two *Staphylococcus aureus* isolates.

Compound	Clinical isolate		ATCC 11632	
	MIC	MBC	MIC	MBC
caffeic acid	0.4	0.4	0.1	0.2
chlorogenic acid	0.4	0.4	0.2	0.4
ferulic acid	0.4	0.4	0.2	0.4
morin	0.2	0.4	0.05	0.2
quercetin	0.2	0.4	0.2	0.4
isoquercitrin	0.2	0.4	0.1	0.2
rutin	0.4	0.8	0.2	0.4
hesperidin	0.4	0.4	0.2	0.4
amox./clav. acid[a]	0.001	0.002	0.001	0.002

[a] Amoxicillin with clavulanic acid are commercial antibiotics used as positive control

3. RESULTS AND DISCUSSION

Antibacterial activities of eight tested phenolic compounds against two strains of the *S. aureus*, are presented in Table 1. The antibacterial activity was in the range of 0.05–0.4 mg/mL for inhibitory activity (MIC values), and 0.1–0.8 mg/mL for bactericidal activity (MBC). *S. aureus* ATCC 11632 was more susceptible to the application of examined phenolic compounds compared to *S. aureus* clinical isolate, MIC in range 0.05-0.2 mg/mL and 0.1-0.4 mg/mL, respectively. Morin, caffeic acid and isoquercitrin showed the best activity against *S. aureus* ATCC 11632 (MIC values 0.05-0.1 mg/mL). Against *S. aureus* clinical isolate morin, quercetin and isoquercitrin were the compounds with the best activity (MIC value 0.2 mg/mL). Commercially available antibiotic amoxicillin with clavulanic acid proved to be more effective against *S. aureus* isolates than the phenolic compounds, but considering its side effects and raising antimicrobial resistance among pathogens the activity of naturally bioactive compounds is of great importance. In the past few years, there are several reports regarding antibacterial activity of caffeic acid (dos Santos et al., 2018; Lima et al., 2016; Kępa et al., 2018), chlorogenic acid (Adamczak et al., 2019; Li et al., 2014; Wang et al., 2015), ferulic acid (Borges et al., 2013; 2012), morin (Gutiérrez-Venegas et al., 2019), quecetrin (Adamczak et al., 2019; da Costa Júnior et al., 2018; Gutiérrez-Venegas et al., 2019), hesperidin (Lopes et al., 2017), and rutin (Gutiérrez-Venegas et al., 2019) against *S. aureus* different strains (Table 2).

Biofilm results indicated that among eight tested phenolic compounds, ferulic acid, morin, quercetin, and hesperidin inhibit

Table 2. Previously reported minimal inhibitory concentrations (MIC) of selected phenolic compounds against different *Staphylococcus aureus* isolates

Compound	Isolate	MIC [mg/mL]	Reference
caffeic acid	Clinical isolate	≥1.024	Li et al. (2014)
	ATCC 25923, ATCC 43300, ATCC 6538	0.256	Kępa et al. (2018)
	RN4220, 1199B	>1.024	dos Santos et al. (2018)
chlorogenic acid	ATCC 29213	2.56	Li et al. (2014)
	ATCC 25904, ATCC 25923, ATCC 29213	>1.024	Wang et al. (2015)
	unknown	1.000	Adamczak et al. (2019)
ferulic acid	CECT 976	1.100	Borges et al. (2013; 2012)
morin	ATCC 25923	1.000	Gutiérrez-Venegas et al. (2019)
quercetin	Clinical isolates, ATCC 29213, ATCC 33591	0.001 – >0.256	da Costa Júnior et al. (2018)
	ATCC 25923	0.050	Gutiérrez-Venegas et al. (2019)
	unknown	> 1.000	Adamczak et al. (2019)
hesperidin	RN4220, SA1199B	>1.024	Lopes et al. (2017)
rutin	ATCC 25923	1.000	Gutiérrez-Venegas et al. (2019)

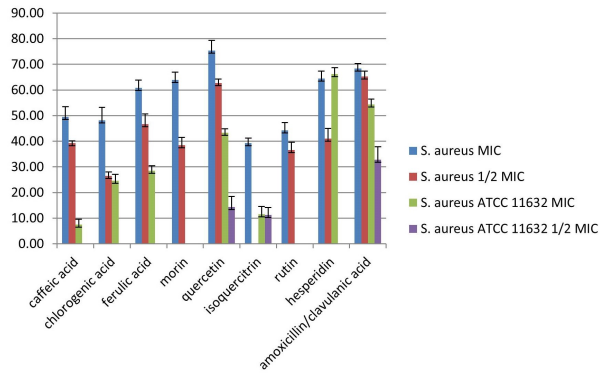

Fig. 1. Inhibition of *S. aureus* (clinical isolate) and *S. aureus* ATCC 11632 biofilm formation by phenolic compounds (%).

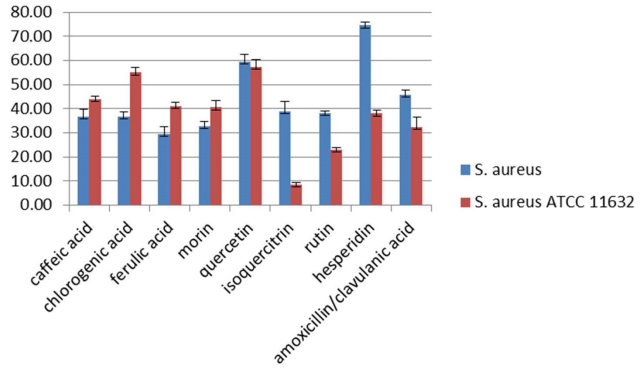

Fig. 2. Destruction of 24 h old *S. aureus* (clinical isolate and ATCC 11632) biofilms after 60 s treatment with MBC of phenolic compounds (%).

S. aureus (clinical isolate) cell attachment for above the 60 % at MIC value, while for *S. aureus* ATCC 11623, only hesperidin showed inhibition of biofilm formation above 60 % (Figure 1). Among the tested compounds, caffeic acid showed the lowest antibiofilm activity with an inhibition value of 7.5 % (for *S. aureus* ATCC 11632) and isoquercitrin with 39 % (for *S. aureus* clinical isolate). Isoquercitrin didn't exhibit antibiofilm activity towards *S. aureus* clinical isolate at sub-MIC value. Morin and rutin are two compounds which didn't show antibiofilm activity towards *S. aureus* ATCC 11632. Caffeic, chlorogenic, ferulic acids and morin, rutin and hesperidin didn't exhibit antibiofilm effect on *S. aureus* ATCC strain at sub-MIC (Figure 1). The effect of the 60 s treatment with different phenolic compounds on *S. aureus* established biofilms was tested. All examined compounds exhibited demolishing activity against established biofilms (Figure 2). The best activity was accomplished by hesperidin its application has reduced established biofilm biomass of *S. aureus* clinical isolate for 74 %, while quercetin demolished *S. aureus* ATCC 11632 biofilm for 57 %. Isoquercitrin showed the lowest activity among the tested natural products. According to Cho et al. (2015), quercetin at 0.05 mg/mL significantly inhibits biofilm production of *S. aureus* ATCC 6538 strain after 24 h while in our study concentration of 0.2 mg/mL quercetrin was necessary for biofilm reduction. Abreu et al. (2016) demonstrated effect of the morin and quercetin in biofilm prevention, when they were applied alone or in combination with antibiotics. Morin (500 mg/L) applied for 1 h caused high CFU reduction (1.2-2.1 CFU cm^2) than quercetrin and antibiotics, but after 24 h, both morin and quercetrin showed activity in preventing biofilm formation of SA1199B and XU212 strains. In the combination, tetracycline combined with pyrrolidine and morin showed promising effect against XU212 strain.

Phenolic acids have shown the similar behaviour in microdilution test against free floating cells and against microbial biofilm. It could be presumed that glycosylation of flavonoids (quercetin as aglycon) contributes to the lower ac-

tivity recorded in the tests of antibiofilm activity. However, glycosylation of flavonoids seemed to have better or no effect on MICs than aglycone forms, depending on substitution position of sugars, as shown by the results of microdilution method. According to Méndez and Salas (2001) and Hyung Ko et al. (2006), sugar ligands can mediate drug targeting and biological activity. Overall, glycosylated molecules were less active against microbial biofilms, and their activity seemed to be time dependant. Overall, all of the phenolic compounds expressed the activities investigated in the current study. It seems that antimicrobial potential of phenolic acids versus flavonoids was subordinated to chemical structures of individual compounds.

CONCLUSION

The results obtained in this work indicate that phenolic compounds, particulary morin possess promising antibacterial activity. Furthermore, all tested compounds exhibited promising antibiofilm potential in both assays - feature especially accomplished by quercetin and morin. The observed antimicrobial and antibiofilm potential of phenolic compounds can serve as one step more towards the development of novel treatment for the *S. aureus* infections.

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