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New insights into the underlying mechanism involved in the *Frangula alnus* antivirulence potential directed toward *Staphylococcus aureus*

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ABSTRACT:
Staphylococcus aureus is a leading cause of persistent infections which are difficult to treat due to its biofilm formation capability and increased resistance to available drugs. Our previous research showed the high antibiofilm potential of the ethyl-acetate extract of *Frangula alnus* and its main component emodin against *S. aureus* so this study was conducted to elucidate the mechanism behind the observed activity. The main goal of this research was to examine the inhibitory effect of *F. alnus* ethyl-acetate extracts and emodin on *S. aureus* biofilm matrix components (exopolysaccharides and eDNA), persister cells, and the staphyloxanthin pigment. It was demonstrated that both substances significantly reduced the production of exopolysaccharides and the amount of eDNA, and decreased the number of persister cells in the studied strains and isolates. However, the effect on staphyloxanthin production was less pronounced, with emodin being more effective. Based on the obtained results, it could be concluded that both the ethyl-acetate extract of *F. alnus* and emodin are good candidates for novel antibiofilm agents acting on *S. aureus* biofilm at different levels.

Keywords: extracellular matrix, persister cells, *Staphylococcus aureus*, staphyloxanthin

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INTRODUCTION

Infections caused by biofilm-forming pathogens ate the leading cause of patient therapy failure, resulting in high mortality rates (Singh *et al.* 2022). Biofilm architecture enables survival in unfavourable conditions, and confers tolerance to antimicrobial agents due to certain biofilm characteristic such as the presence of an extracellular matrix (ECM) and persister cells (Arciola *et al.* 2018). In most biofilms, the ECM constitutes up to 90% of the dry mass, while the remaining 10% is made up of bacterial cells (Das 2022). The ECM surrounds the cells, keeping them close and providing them with mechanical stability, a complex chemical microenvironment and enhanced interaction opportunities, which are all necessary for the biofilm lifestyle. Additionally, this type of organisation also contributes to increasing resistance to antimicrobial agents including antibiotics and the host immune system (Karygiann *et al.* 2020). The ECM is mainly composed of extracellular polysaccharides

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(EPS), proteins, lipids, and nucleic acids (eDNA), with EPS and proteins being the dominant and the most studied components. Furthermore, biofilms also contain large amounts of eDNA, which was initially thought to be a remnant of lysed cells, but has recently been recognised as an important ECM component (Molin & Tolker-Nielsen 2003).

Another important characteristic of biofilm is the presence of persister cells, a subpopulation of dormant cells resistant to high concentrations of antibiotics, as well as to the host immune system mainly due to their dormancy and/or inaccessibility (Lewis 2001; Defraine *et al.* 2018). Persister cells make up about 0.1–10% of the biofilm and represent promising target sites for the action of antimicrobial agents with the aim of controlling biofilm-induced infections, as well their spread (Percival *et al.* 2011). Their number depends on the growth phase and can increase not only in response to high doses of antibiotics, but due to a lack of nutrients within the biofilm, high pH, and damage to DNA molecules (Defraine *et al.* 2018). The survival of persister cells could be linked to their ability to be metabolically inactive and to 'turn off' antibiotic targets (Stewart & Franklin 2008). After antibiotic treatment, these cells return to the growth state and could reestablish the infection.

Among numerous human pathogens, *Staphylococcus aureus* is one of the major causes of community-acquired, nosocomial, and biofilm-related infections worldwide (Sures *et al.* 2019). Biofilm-related infections caused by this pathogen are very difficult to treat due to its high resistance to antimicrobials, and the production of a wide range of virulence factors which contribute to its significant pathogenic efficiency (Mulcahy & McLoughlin 2016; Parastan *et al.* 2020; Yang *et al.* 2022). From the various virulence factors in *S. aureus*, one stands out in particular: its golden, carotenoid pigment staphyloxanthin (Liu & Nizet 2009). Staphyloxanthin acts as an antioxidant, capable of scavenging the free radicals secreted by lysosomes and consequently providing resistance to immune defence. Furthermore, it plays a key role in regulating the mechanical properties of the cell membrane maintaining the integrity of bacterial cell membrane while simultaneously limiting the host's oxidative defence mechanisms (Mishra *et al.* 2011).

It is clear that infections caused by *S. aureus* biofilms are hard to treat and that one of the alternative ways to combat this pathogen is to act on new targets, such as ECM or staphyloxanthin production. Since this pathogen is often resistant to multiple conventional antibiotics, natural products including plant extracts and pure compounds may potentially provide plausible alternatives/adjuncts. Plants are rich in secondary metabolites which possess numerous biological activities, making them good candidates for alternatives to available therapeutics (Jadimurthy *et al.* 2023). Consequently, this study builds on our previous research on the antibiofilm activity of *Frangula alnus* bark and its main constituent emodin on *S. aureus* biofilms. Concerning emodin activity (1,3,8-trihydroxy-6-methylanthraquinone), it is well known that the group of anthraquinones (including emodin) possess antibacterial potential. The structure of the anthracene ring could be considered as the core skeleton for its biological activity. Furthermore, the polar substituents, including the –OH group of emodin, could be considered as contributing factors for improving the antibacterial activity of the anthraquinone core (Qun *et al.* 2023). Although certain biological activities of plants of the *Frangula* genus, such as antifungal, antibacterial, antioxidant and anticancer have been previously demonstrated (Manojlović *et al.* 2005; Kremer *et al.* 2012; Brkanac *et al.* 2015; Azadkhah & Farahani 2016), our research group has recently focused on their antibiofilm activity. Our previous studies (Đukanović *et al.* 2020, 2022) have shown that the ethyl-acetate extract of *F. alnus,* and its dominant constituent emodin, exhibited extraordinary antistaphylococcal activity, especially regarding the bacterial biofilm. Both substances inhibited biofilm for-

mation, disrupted preformed biofilm in several clinical isolates of *S. aureus*, and modulated the gene expression of *ica*A, *ica*D, *srr*A, and *srr*B, all of which are involved in biofilm formation, as well as the expression of micro RNA-RNA III. In addition, they also affected, mainly inhibited, bacterial aerobic respiration. Therefore, in order to further clarify the antibiofilm mechanism, the aim of this study was to examine the effect of the ethyl-acetate extract and its dominant constituent emodin on the ECM of *S. aureus* biofilm, namely on EPS, eDNA and persister cells. Staphyloxanthin production was also studied, since it is considered a key virulence factor in *S. aureus* pathogenesis.

MATERIAL AND METHODS

Bacterial strains and growth conditions. The *Staphylococcus aureus* strains used in this study are presented in Table 1. The clinical isolates and growth conditions were previously described in Đukanović *et al*. (2020).

***Frangula alnus* ethyl-acetate extract and emodin.** The ethyl acetate extract of *F. alnus* (FA) was prepared as previously described by Đukanović *et al*. (2020) and the working stock (50 mg/mL) was dissolved in a Mueller Hinton broth (MHB) medium, while the emodin (E) powder (Sigma Aldrich, USA) was dissolved in dimethyl sulfoxide (DMSO) (stock 5 mg/mL). Briefly, the dried plant material was macerated with 80% aqueous ethanol (EtOH) for 24 h, with moderate shaking afterwards, the plant material was removed by vacuum filtration and the process was repeated two more times. The extract fractions were collected and the solvent was evaporated to dryness under vacuum at 45°C. The extract was then washed with petroleum ether and the resulting fraction was discarded, while the water fraction was further extracted by ethyl-acetate (EtOAc). The EtOAc extract was evaporated to dryness and dissolved in DMSO to obtain a stock solution (300 mg/mL) from which the working solution was prepared. Both test substances were screened in further tests applying concentrations determined in accordance with previous research (Đukanović *et al*. 2020, 2022). The tested concentrations were different fractions/multiplications of previously determined minimal inhibitory concentration (MIC) values (Table 2), and were applied in accordance with the protocols used in each subsequent test.

Exopolysaccharide extraction from the biofilm. Exopolysaccharide (EpS) extraction from the *S. aureus* strains and isolates biofilm was performed according to Liu *et al*. (2017), with some modifications. Briefly, 1 mL of bacterial suspension (10^6 CFU/mL) in MHB medium (containing 0.5% glucose) was seeded in 12-well plates and incubated for 24 h at 37°C. The next day, the medium was replaced with a fresh containing the treatment at a concentration of 4 × MIC and the incubation was continued for another 24 h. The following day, the medium was removed, the same volume of distilled water (dH_2O) was added, the biofilm was scraped from the bottom of the well and the resulting suspension was centrifuged for 10 min at 12,000 rpm (Hettich zentrifugen, Micro 22R, Germany). The supernatant was transferred to new tubes, the remaining pellet was washed with additional dH_2O and the process was repeated. In this way, the water-soluble glucans (WSG) were separated. The precipitate obtained was resuspended in 1 mL of 0.1 M NaOH and centrifuged in order to extract water-insoluble glucans (WIG). The supernatant was then separated into new tubes for further centrifugation. Three volumes of ice-cold 96% EtOH were added and incubated at 4°C for 24 h to precipitate the glucans. Afterward, the test tubes were centrifuged for 10 min at 5000 rpm, and the resulting precipitate, containing glucans, was resuspended in 2 mL of dH_2O. For the quantification of EPS, concentrated H_2SO_4 and a cooled 5% aqueous

Table 1. The *Staphylococcus aureus* strains used in this research.

Strains	Origin	Sensitivity
Staphylococcus aureus ATCC 25923	ATCC	MSSA
Staphylococcus aureus ATCC 43300	ATCC	MRSA
Staphylococcus aureus Gp41	Surgical wound	MSSA
Staphylococcus aureus Gp19	Nasal carriage	MSSA
Staphylococcus aureus Gp29	Blood culture	MRSA
Staphylococcus aureus Gp7	Nasal carriage	MRSA

Table 2. Previously determined minimal inhibitory concentrations (MIC) values for the ethyl acetate extract of *Frangula alnus* (FA) and emodin (E) (Đukanović *et al*. 2020, 2022).

Staphylococcus aureus strains	FA	E
	MIC (μg/mL)	
Staphylococcus aureus ATCC 25923	500	25
Staphylococcus aureus ATCC 43300	62.5	12.5
Staphylococcus aureus Gp41	125	12.5
Staphylococcus aureus Gp19	62.5	6.25
Staphylococcus aureus Gp29	62.5	3.125
Staphylococcus aureus Gp7	125	3.125

solution of phenol, in a volume ratio of 1:5:1, was added to the test tubes. The test tubes were then incubated for 5 min at 90°C in a water bath. After cooling, the absorbance was measured at 492 nm.

eDNA extraction. The extraction of eDNA from the biofilms was performed according to Wu & Xi (2009) with slight modifications. The biofilms were grown as described in the previous section and treated with concentrations of 4 × MIC of both substances. After incubation they were peeled off with 100 μL of 1 × PBS. The mass was transferred to tubes with proteinase K (final concentration 5 μg/mL) and incubated for 2 h at 37°C. Following incubation, the tubes were centrifuged at 12,000 rpm for 10 min and the supernatant was collected. eDNA was extracted by ethanol precipitation, by adding 2.5 volumes of 100% EtOH and 1/10 volume of 3M Na-acetate and centrifugation (12,000 rpm, 10 min). The resulting supernatant was removed, the tubes were dried at room temperature, and the precipitate was resuspended in 15 μL of TE buffer. The extracted eDNA quantity was determined spectrophotometrically using a BioSpec-nano (Schimadzu Corporation, Kyoto, Japan).

Isolation of the persister cells from the biofilms. The isolation of the persister cells from the biofilms followed the method of Willenborg *et al*. (2014), with some modifications. To monitor the effect of the test substances on the number of persister cells, the biofilms were prepared as described above. In this experiment, 100 × MIC concentrations of the test substances were used for the treatment. The biofilm was removed from the bottom of the well by adding 500 μL

of 1 × PBS and peeling off the mass. The peeled mass was centrifuged at 12,000 rpm for 5 min. The supernatant was removed, and the obtained precipitate was resuspended in 500 µL of 1 × PBS. Serial dilutions were prepared from the suspension and 100 µL of the desired dilution was plated on LA substrate and incubated for 24 h at 37°C. After incubation, the colonies were counted and the number of CFU/mL was calculated. The number of persisters from the treated biofilm was compared to those from the untreated biofilm.

Isolation of staphyloxanthin. Pigment isolation was performed as described by Barretto & Vootla (2018). Overnight cultures of bacteria were diluted to10^8 cells/mL and 100 µL of inoculum was seeded onto Triptoc Soy agar (TSA) plate containing ×MIC of the test substance, as well as onto un-supplemented plates used as the control. The plates were incubated for three days at 37°C, then for two days at 20°C. After incubation, all the colonies were removed and resuspended in 1 mL of dH_2O. The agar surfaces were additionally washed with 2 mL of dH_2O, which was added to the previously resuspended colonies. The test tubes were centrifuged for 15 minutes at 5000 rpm, and the supernatant was discarded. The precipitate was then resuspended in 1 mL of dH_2O and the centrifugation was repeated. The obtained precipitate was resuspended in 3 mL of absolute methanol and the test tubes were heated for 5 min at 55°C. The cooled tubes were centrifuged again for 15 min at 5000 rpm. The absorption spectrum for the obtained supernatant was measured in the wavelength range of 300–600 nm using a spectrophotometer (UV-1280, Shimadzu, Japan) with the staphyloxantine peak at 460 nm. The percentage of inhibition was calculated according to following formula:

% of inhibition =1-(A460nmC/A460nmT)* 100

$A_{460nm}C$ - absorption peak of staphyloxantine at 460nm in the controls

$A_{460nm}T$ - absorption peak of staphyloxantine at 460nm in the treatments

Statistical analysis. Statistical processing of the results was done using the GraphPad Prism 6.01 software program (Software, Inc., USA). The data were subjected to one-way analysis of variance (one-way ANOVA) using Dunnett's test. The threshold of statistical significance was set at $p < 0.05$, $p < 0.01$, and $p < 0.001$. All the experiments were conducted at least three times, with each test performed in triplicate.

RESULTS

The effect of FA and E on EPS production. The influence of the test substances on the EPS production in the biofilm matrix was monitored using the phenol-sulfuric acid technique. Based on the obtained results (Fig. 1), it can be observed that FA inhibited the production of WSG in *S. aureus* ATCC 25923 (69%) and *S. aureus* ATCC 43300 (76%) strains and isolate Gp19 (75%). Similar results were observed for E with the highest inhibition demonstrated in the Gp19 isolate (79.9%). The effect of the substances on WIG varied depending on the treatment type (FA or E) and was strain/isolate-specific. The most notable was the effect of FA on ATCC 43300 (43% inhibition, Fig. 1D). In contrast, the FA extract induced a significant increase of WIG in the Gp7 strain. When it comes to E, a slight inhibition of synthesis occurred in isolates Gp29 and Gp7 (Fig. 1E and 1F).

The effect of FA and E on eDNA. The results showing the effect of FA and E on the concentration of eDNA are presented in Table 3, indicating strain, and isolate- and treatment-specific activity. The amount of eDNA in the matrix increased in treatments with both FA and E in the ATCC 25923 and Gp41 strains, as well as in the cases of FA-treated Gp29 and E-treated Gp7. Con-

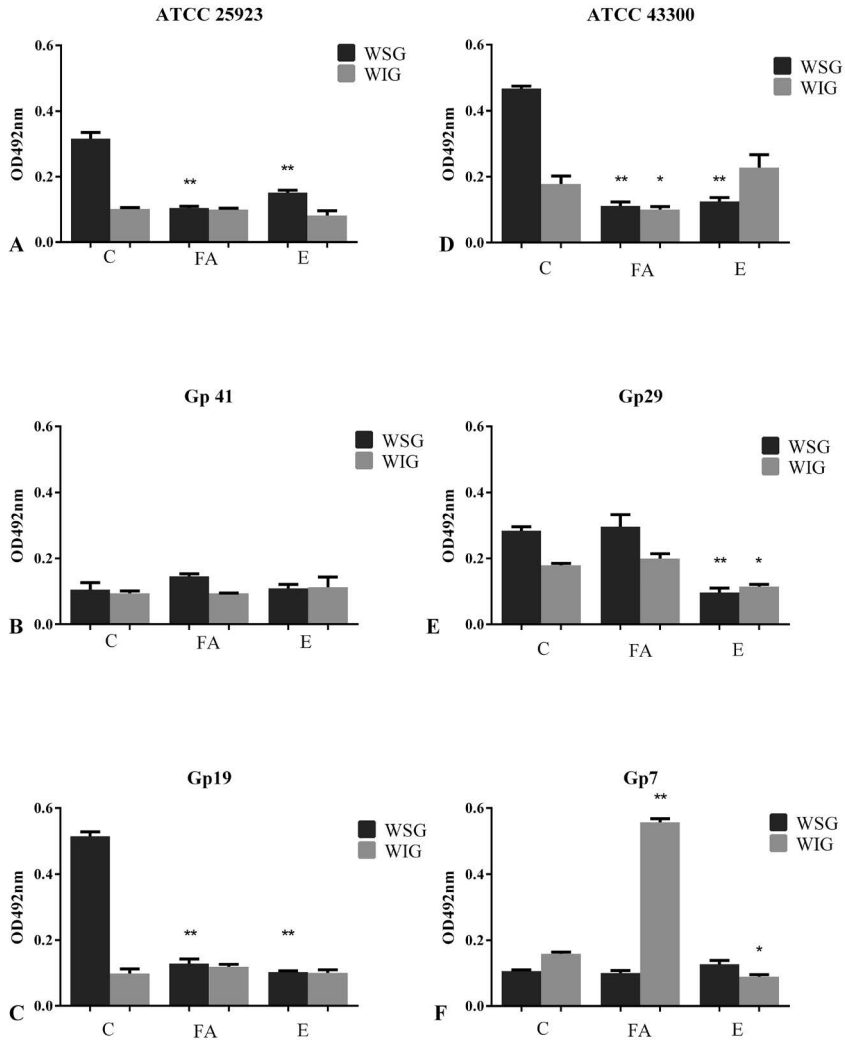

Fig. 1. The effect of the *Frangula alnus* extract and emodin on the extracellular matrix polysaccharides of *S. aureus* biofilms. C-control; FA-treatment with the *Frangula alnus* extract; E-treatment with emodin; WSG-water soluble glucans; WIG-water insoluble glucans. The results are presented as means ± standard deviations. Statistical differences in respect to the un-treated control: * $p < 0.05$, ** $p < 0.01$.

Table 3. The concentrations of the obtained eDNA in the matrix of *Staphylococcus aureus* biofilms.

	eDNA (ng/μL)					
	Staphylococcus aureus					
Strains	ATCC 25923	Gp41	Gp19	ATCC 43300	Gp29	Gp7
Control	1.81±0.24	12.2±1.66	39.1±0.20	25.67±1.53	43.33±1.20	20.7±1.32
FA	3.5±0.36*	16.73±0.57**	35.87±1.53*	16.67±0.78**	54±2.65*	14.33±0.60*
E	6.57±0.59**	26.03±0.86**	16.3±0.46**	13.3±0.46**	39.16±0.31**	28.66±0.45**

FA-treatment with *Frangula alnus* extract; E-treatment with emodin. Statistical difference in respect to untreated control: * $p<0.05$, ** $p<0.01$.

Table 4. The number of persister cells in untreated and treated biofilms of the *Staphylococcus aureus* strains.

	Control	FA	E
		log CFU/mL	
Staphylococcus aureus ATCC 25923	8.78±0.07	8.10±0.10**	7.95±0.06**
Staphylococcus aureus Gp41	8.85±0.09	7.84±0.05**	7.93±0.04**
Staphylococcus aureus Gp19	8.38±0.05	8.44±0.08	8.19±0.12
Staphylococcus aureus ATCC 43300	9.21±0.09	8.42±0.03**	8.51±0.08**
Staphylococcus aureus Gp29	8.73±0.04	8.45±0.07*	8.65±0.04
Staphylococcus aureus Gp7	8.73±0.06	8.75±0.05	8.58±0.18

FA-treatment with *Frangula alnus* extract; E-treatment with emodin. Statistical difference in respect to un-treated control: * $p<0.05$, ** $p<0.01$.

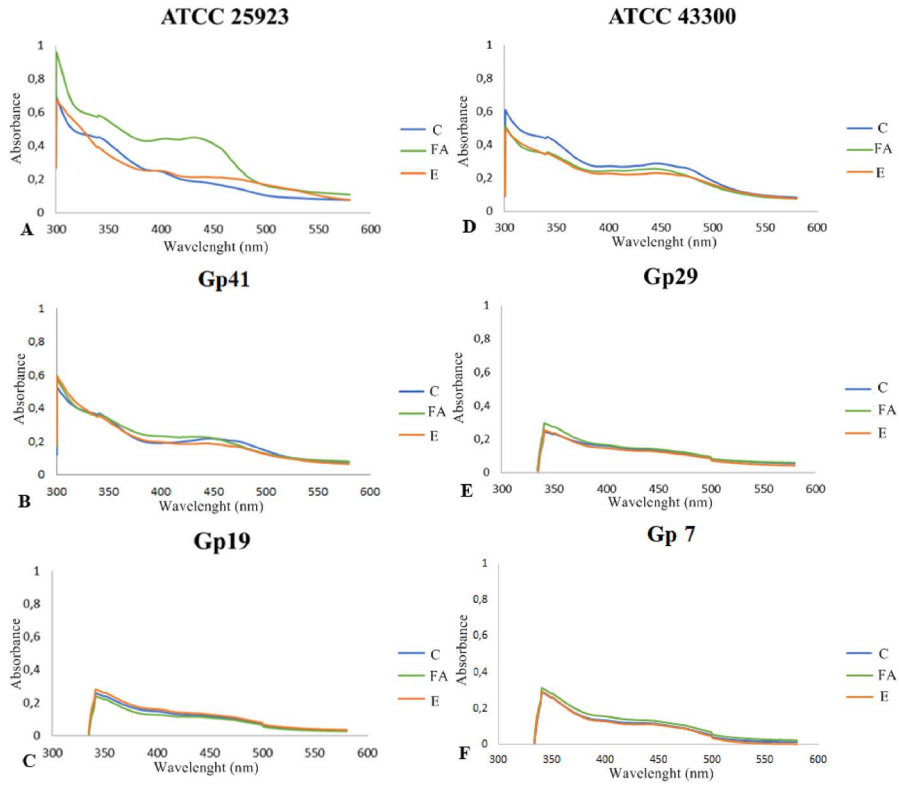

Fig. 2. Absorption spectra of staphyloxanthin under the influence of the FA extract and emodin in the *Staphylococcus aureus* strain. C-control; FA-treatment with the *Frangula alnus* extract; E-treatment with emodin.

versely, a reduction in eDNA concentration was observed in the case of both treatments in Gp19 and ATCC 43300, and additionally in E-treated Gp29 and FA-treated Gp7.

The effect of FA and E on the persister cells in the biofilms. The effect of the test substances on the number of persister cells was monitored indirectly, and the results are shown in Table 4. The obtained values show that in most cases

Table 5. Inhibitions (%) of staphyloxanthin production in the *Staphylococcus aureus* strains treated with the *Frangula alnus* extract and emodin.

	FA	E
	% of inhibition	
Staphylococcus aureus ATCC 25923	nd	nd
Staphylococcus aureus Gp41	nd	14.42
Staphylococcus aureus Gp19	7.64	nd
Staphylococcus aureus ATCC 43300	11.39	19.97
Staphylococcus aureus Gp29	nd	6.88
Staphylococcus aureus Gp7	nd	13.72

FA-treatment with *Frangula alnus* extract; E-treatment with emodin; nd-not determined

FA and E led to a statistically significant decrease in the number of persister cells in the biofilm. The only exceptions were Gp7 and Gp19 treated with FA.

The effect of FA and E on staphyloxanthin production. The results of staphyloxanthin biosynthesis are given in Fig. 2. The obtained absorption spectra showed that both test substances had a weak effect on staphyloxanthin pigment synthesis. The only exception was the effect of FA on the strain ATCC 25923 where its absorbance curve was notably higher compared to that obtained for control, indicating higher production of staphyloxanthin. In contrast, the highest percentage of inhibition, approximately 20%, was recorded in the ATCC 43300 strain treated with E (Table 5).

DISCUSSION

In recent years resistance to conventional antibiotics has emerged as a serious problem facing medicine and humanity, emphasising the urgent need to address it. Antivirulence compounds have an important place in the search for new strategies in the fight against resistance These compounds suppress the virulence factors responsible for pathogenicity, including the formation of biofilm (Xue *et al*. 2019). An aggravating circumstance is the ability of bacteria to form biofilms, thus leading to serious systemic infections (Sacco *et al*. 2020). Moreover, biofilm is one of the main virulence factors of *S. aureus* strains, responsible for persistent infections and complicating treatment. As the effectiveness of available drugs continues to decline, a great deal of research is directed towards finding new natural agents and strategies, as well as ways to improve the efficacy of existing ones. Therefore, plants attract special attention due to the variety of secondary metabolites and their derivatives and the numerous biological activities they possess (Farha & Brown 2015). In our previous research, *F. alnus* was chemically analysed and E was singled out as the dominant component of FA (Đukanović *et al*. 2020), and consequently included in further research (Đukanović *et al*. 2022). In addition to antibacterial activity, the substances showed strong antibiofilm activity against *S. aureus* strains and isolates. Briefly, FA and E inhibited biofilm formation and disrupted preformed biofilm, acted on the expression of selected genes involved in the biofilm formation control, as well as on aerobic respiration as previously shown (Đukanović *et al*. 2022). However, the molecular mechanism(s) were left to be further elucidated, and since the important biofilm matrix compo-

nents are EPS and eDNA (Di Martino 2018; Mishra *et al.* 2020), those were of prime interest for research. Given the importance of persister cells for biofilm vitality maintenance, as well as the crucial role of staphyloxanthin as a virulence factor, those were also included in the research.

As previously mentioned, biofilm cells are immersed in an organic extracellular matrix which they produce themselves and is involved in their interaction with the environment. Numerous antibiofilm compounds precisely target the biofilm matrix, whose disintegration could lead to the exposure of cells to antimicrobials which act successfully against planktonic bacteria (Mishra *et al.* 2020). The destabilisation of WSG and WIG would allow more successful treatment of biofilm-associated infections. WSG can serve as a source of energy and nutrition in adverse conditions, but can also contribute to adhesion, while WIG dominantly contribute to biofilm stability and enable adhesion (Liu *et al.* 2017; Nguyen *et al.* 2020). Based on the results obtained in this study, it can be observed that the inhibitory effect of FA and E was more pronounced on WSG in *S. aureus* biofilms. In addition, the effect of FA and E on WIG was extremely weak or did not result in significant changes. In line with these results, Emeka *et al.* (2020) demonstrated that the natural compound mangiferin (from the plant *Mangifera indica*) reduced WSG levels in the biofilm of *S. mutans*. Conversely, Liu *et al.* (2019) showed that surfactin could prevent biofilm formation by acting on WIG in the *S. mutans* strain. However, contrary to the existing literature, in this study, an additional production of insoluble glucans was observed in certain strains and isolates with certain treatments. The obtained inhibitory effect of the test substances on WSG combined with the absence of any effect on WIG, or even their increase, may indicate a redistribution of the necessary energy within the biofilm. We assume that bacteria in the biofilm tend to protect themselves from external agents and stressors, so there is a possibility that during the treatment with our substances energy was redirected to the production of WIG, since they are responsible for the stability of the biofilm. Additionally, the stimulatory effect on EPS production can be explained by stress induction caused by the test substances, leading to the increased production of insoluble polysaccharides, as was previously postulated by Landini (2009). This fact is supported by their previously mentioned contribution to biofilm stability.

Another important component in the *S. aureus* biofilm matrix is eDNA, whose presence in ECM may result from cell lysis or active shedding (Schwartz *et al.* 2016). It can interact with other components of the matrix, contributing to the biofilm structure and bacterial defence against antimicrobial agents, and can also play a role in biofilm formation (Karygianni *et al.* 2020). In this study, the effect of FA and E on eDNA concentration was also investigated. Both test substances led to decreased amounts of eDNA in ATCC 43300, Gp19, Gp29 (E treatment) and Gp7 (FA treatment). Similar results were obtained in the study carried out by Yan *et al.* (2017), where there was a significant decrease in eDNA concentration in *S. aureus* biofilm treated with emodin. The reduction in the amount of eDNA in the matrix affects the sensitivity of the biofilm as this component contributes to adhesion as well as protection against antimicrobial agents. However, this effect was not beneficial in all the tested strains and isolates. Conversely, an increase in the amount of eDNA after treatment with FA and E was observed in certain strains and isolates. Similarly, Mlynek *et al.* (2016) showed that exposure to amoxicillin also led to an increased presence of eDNA in the *S. aureus* matrix. Furthermore, Doroshenko *et al.* (2014) reported an increase of eDNA concentration in the biofilm of *Staphylococcus epidermidis* after treatment with methicillin and vancomycin. In these studies, it was found that the tested substances led to cell lysis, which consequently results in an increase of eDNA. Therefore, the detected increase in the amount of eDNA in ATCC 25923, Gp41 and Gp7

could be explained by this fact. Contrary to the above data, Xiang *et al.* (2017) showed that aloe-emodin had no effect on the amount of eDNA in the biofilm of *S. aureus* strains.

An interesting and important target in the fight against biofilms is persister cells. It is clear that reducing the number of these cells in the biofilm is crucial in treating infections caused by bacterial biofilms. Thus, the effect of FA and E on the number of persister cells was further investigated. The results obtained indicate a decrease in the number of persister cells in the majority of strains. In the study conducted by Orman & Brynildsen (2015), it was shown that certain compounds, such as nitric oxide, possess the ability to reduce persister cells in *Escherichia coli*. In their research, Ghosh *et al.* (2015) showed that aryl-alkyl-lysines can reduce the number of persisters in MRSA strains of *S. aureus*. Yang *et al.* (2018) demonstrated that emodin modulates the cell membrane permeability of *S. aureus* strains, suggesting that it acts in the same way on persister cells, since in some strains and isolates it led to a decrease in their number in preformed biofilms. However, in the obtained results, in contrast to other strains, there was no effect of either substances in the isolates Gp19 and Gp7. The explanation for this phenomenon may lie in the fact that the stressful conditions which inhibit bacterial growth may induce increased persistence, resulting in a balance between newly formed persisters and those reduced by treatment. Additionally, it is known that persistence can occur spontaneously (Kaldalu *et al.* 2020).

One of the key virulence factors of *S. aureus* is their carotenoid pigment staphyloxanthin, which acts as an antioxidant and protects the cells against the negative impact of ROS produced by host neutrophils (Ni *et al.* 2018). Considering the fact that inhibiting pigment synthesis could render the cells more sensitive to the effects of therapeutic agents, the effect of FA and E on staphyloxanthin production was also examined. Their effect on pigment production was very weak, and was most pronounced in the strain ATCC 43300. However, although a great deal of data indicates the ability of natural compounds to inhibit staphyloxanthin production, in most of our strains and isolates the test substances were not particularly successful, and in some cases they even caused additional pigment production. Although the findings in this study are quite variable and strain- and isolate-specific, they could provide an important starting point for further more detailed analysis aimed at potentially developing a novel antistaphylococcal agent.

In addition, our previous work explored the cytotoxic and genotoxic potential of FA and E (Vuletić *et al.* 2023). The obtained results of cytotoxicity testing using the MTT assay on the MRC-5 cell line indicate the safety of using these substances. On the other hand, at the two highest tested concentrations, the effect of both substances on cell cycle, cell death and mitochondrial membrane potential was recorded using flow cytometry analysis, as well as their genotoxicity using the comet assay. However, it is important to point out that the concentrations of FA and E in these two studies are not the same. Thus, this potential problem could be overcome by selecting appropriate doses.

CONCLUSIONS

To the best of our knowledge, this is the first study investigating the effect of the *Frangula alnus* ethyl-acetate extract and its dominant component emodin on biofilm matrix components (extracellular polysaccharides and eDNA), persister cells and pigment staphyloxanthin in selected *Staphylococcus aureus* referent strains and clinical isolates. The effects of both substances on the selected targets were variable and strain-specific. According to the results obtained, both FA and E exhibit inhibitory potential against certain components of the *S. aureus* biofilm matrix. Both substances decreased the number

of water-soluble polysaccharides and eDNA in certain strains and isolates. In addition, they reduced the quantity of persister cells in the biofilms, at least in most of the tested strains. Taking into account the findings from our previous study indicating notable antibiofilm activity, it can be concluded that both substances act through multiple pathways and levels on *S. aureus* biofilm. However, despite their pronounced antibiofilm effect, their impact on the pigment was negligible. Summarising the results obtained across all the tests, the MRSA strain ATCC 43300 was the most sensitive to the *F. alnus* ethyl-acetate extract and emodin, with emodin showing a more pronounced effect. However, based on the findings, we can conclude that further research involving a larger number of isolates and a deeper investigation of the underlying mechanisms is needed in order to make a reliable assessment of the efficacy against *S. aureus* strains and to define the precise mechanisms of action, aiming to optimally utilize their antibiofilm potential.

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COMPREHENSIVE PLANT BIOLOGY

REZIME

Novi uvid u mehanizme u osnovi antivirulentnog potencijala *Frangula alnus* usmerenog ka *Staphylococcus aureus*

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Staphylococcus aureus je vodeći uzročnik infekcija koje se teško leče i to zbog sposobnosti formiranja biofilma i povećane otpornosti na dostupne lekove. U našim prethodnim istraživanjima pokazan je visok antibiofilm potencijal etil-acetatnog ekstrakta *Frangula alnus* i njegove glavne komponente emodina prema *S. aureus*, te je ova studija sprovedena upravo da bi se rasvetlio mehanizam zabeležene aktivnosti. Osnovni cilj ovog istraživanja bio je ispitivanje inhibitornog dejstva etil-acetatnog ekstrakta *F. alnus* i emodina na komponente matriksa biofilma *S. aureus* (egzopolisaharidi i eDNK), ćelije perzistere u biofilmu i njegovog pigmenta-stafiloksantina. Pokazano je da obe supstance uglavnom smanjuju proizvodnju egzopolisaharida i količinu eDNK u biofilmu, kao i da smanjuju broj ćelija perzistera kod nekih istraživanih sojeva. Međutim, efekat na proizvodnju stafiloksantina bio je manje izražen, pri čemu se emodin pokazao kao efikasniji. Na osnovu dobijenih rezultata, možemo zaključiti da su ekstrakt i emodin dobri kandidati za nove antibiofilm agense koji deluju na biofilm *S. aureus* na različitim nivoima.

Ključne reči: ekstracelularni matriks, ćelije persistere, *Staphylococcus aureus*, stafiloksantin