INTRODUCTION

In the last decades antibiotics have been widely and irrationally used and that led to a global problem of bacterial resistance these days and consequently to a necessity for new antimicrobials. Moreover, novel approaches and strategies that could be considered successful in resolving this problem are urgent due to the growing mortality rate caused by bacterial resistance (Ghosh et al., 2019). Therefore, the search for alternative antimicrobial agents among natural products, particularly among medicinal plants, has become intensive.

Plants represent an interesting source of novel therapeutic agents since they are rich in different groups of secondary metabolites and rarely exhibit side effects (Manandhar et al., 2019). In addition, large chemical diversity of secondary metabolite groups is available in nature and consequently they possess numerous biological activities (de Oliveira Galvão et al., 2020). Anthraquinones, which are characterized by great diversity and have found applications in industry and pharmacy, attract attention in particular (Malik & Müller, 2016). Emodin is a common derivative of anthraquinones, and it is a dominant constituent of many medicinal plants, mainly belonging

Abstract

Aims: Because the Staphylococcus aureus is one of the most well-known pathogens associated with medical devices and nosocomial infections, the aim of the study was to examine antibiofilm potential of emodin against it.

Methods and Results: Antibacterial activity was examined through microdilution assay. Antibiofilm testing included crystal violet staining of biofilm biomass and morphology analysis by Atomic force microscopy (AFM). Furthermore, aerobic respiration was monitored using the Micro-Oxymax respirometer. For investigation of gene expression qRT-PCR was performed. Emodin demonstrated strong antibacterial activity and ability to inhibit biofilm formation of all tested strains. The effect on preformed biofilms was spotted in few strains. AFM revealed that emodin affects biofilm structure and roughness. Monitoring of respiration under emodin treatment in planktonic and biofilm form revealed that emodin influenced aerobic respiration. Moreover, qRT-PCR showed that emodin modulates expression of icaA, icaD, srrA and srrB genes, as well as RNAIII, and that this activity was strain-specific.

Conclusion: The results obtained in this study indicate the novel antibiofilm activity of emodin and its multiple pathways of action.

Significance and Impact of Study: This is the first study that examined pathways through which emodin expressed its antibiofilm activity.

KEYWORDS

aerobic respiration, AFM, biofilm, emodin, Staphylococcus aureus
to Rhamnaceae, Polygonaceae and Fabaceae families (Izhaki, 2002; Li & Jiang, 2018). It was demonstrated that emodin has various biological activities, such as antibacterial, antiviral, anticancer and anti-inflammatory. Furthermore, anti-allergic and anti-diabetic activities were reported, as well as its toxicity and ability to attenuate many genes expression (Dong et al., 2016). However, the antibiofilm potential of emodin is poorly investigated.

Examination of antibiofilm potential of natural products seems appropriate, especially when it is necessary to develop a new strategy to fight against bacterial resistance. Biofilms represent complex communities of bacteria, being attached to a variety of surfaces and embedded in a self-produced extracellular matrix, which made them more resistant to antibacterial agents (Sharifi et al., 2018). Formation of biofilms is the main virulence factor of the majority of medically important bacteria, including Staphylococcus aureus, gram-positive, facultative anaerobic bacteria with usual carriage sites in pharynx, perineum, anterior nares and skin (Troeman et al., 2019). This species is responsible for a broad spectrum of infections; they are sometimes invasive and often associated with contaminations of medical devices and implants, as well as with infections of surgical wounds, soft tissues. Furthermore, sepsis and nosocomial infections are considered common consequences of staphylococcal infections (Kourtis et al., 2019). The methicillin-resistant (MRSA) strains present an even more serious problem for human health and treatments (Selvaraj et al., 2021).

Considering the fact that biofilm is very important in the staphylococcal life cycle, formation of it is strictly controlled with several regulatory systems and pathways that unite environmental impact and physiological state of the cells (Schilcher et al., 2020). The main component of the matrix and the main adhesive molecule responsible for S. aureus biofilm formation is the polysaccharide intercellular adhesin (PIA) the production of which is under the control of icaADBC operon. Exactly ica is the dominant pathway of biofilm formation, which is regulated by different stressors, such as antibiotics, anaerobic conditions, temperature and osmolarity (Kirmusaoğlu, 2016). Because S. aureus is a facultative anaerobe, ica expression and PIA production are upregulated by the staphylococcal respiratory response regulator (srrAB). This regulatory system reacts to changes in oxygen level and thus regulates the aerobic-anaerobic switch, by adjusting the expression of genes involved in cellular respiration; moreover, it is involved in control of energy homeostasis, carbon flux and virulence (Rudra & Boyd, 2020). In addition, this system modulates activities of regulatory RNA molecule—RNAIII, which is part of staphylococcal agr quorum sensing mechanism.

Bearing in mind everything mentioned above, the aim of this study was to investigate antibiofilm potential and the mechanism of action of emodin derived from Frangula alnus bark on S. aureus strains. The examination has included the monitoring of emodin activity on biofilm formation and disruption, as well as its effect on biofilm structure and roughness, being analysed by atomic force microscopy (AFM). To elucidate the mechanism of action, aerobic respiration under emodin treatment in planktonic and biofilm form was monitored for the first time, using a Micro-Oxymax respirometer. Furthermore, the influence of emodin on expression of icaA, icaD, srrA and srrB genes and micro RNAIII was investigated.

**MATERIAL AND METHODS**

**Bacterial strains and growth conditions**

Staphylococcus aureus strains tested in this study are presented in Table 1. Clinical isolates were previously described by Đukanović et al. (2020).

**Emodin**

Emodin powder from Frangula alnus bark ≥90%, purchased from Sigma Aldrich, was dissolved in dimethyl sulfoxide (DMSO) in a concentration of 5 mg/ml, and it was used for the experiments.

**Antibacterial activity**

Antibacterial activity was monitored in resazurin incorporated microdilution assay, determining the minimal inhibitory concentrations (MICs), as previously described (Đukanović et al., 2020). Briefly, emodin was diluted two-fold in the range of 1.56–200 µg/ml. The experiments were done three times in duplicate.

<table>
<thead>
<tr>
<th>Strains</th>
<th>Origin</th>
<th>Sensitivity</th>
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<tbody>
<tr>
<td>S. aureus ATCC 25923</td>
<td>ATCC</td>
<td>MSSA</td>
</tr>
<tr>
<td>S. aureus ATCC 43300</td>
<td>ATCC</td>
<td>MRSA</td>
</tr>
<tr>
<td>S. aureus Gp41</td>
<td>Surgical wound</td>
<td>MSSA</td>
</tr>
<tr>
<td>S. aureus Gp19</td>
<td>Nasal carriage</td>
<td>MSSA</td>
</tr>
<tr>
<td>S. aureus Gp29</td>
<td>Blood culture</td>
<td>MRSA</td>
</tr>
<tr>
<td>S. aureus Gp7</td>
<td>Nasal carriage</td>
<td>MSSA</td>
</tr>
</tbody>
</table>
Biofilm formation and dispersal assay

Antibiofilm activity of emodin was examined by quantification of biofilm biomass in 96-well microtitre plates by crystal violet (CV) staining. For the effect on biofilm formation, bacterial inoculums in MHB supplemented with 0.5% glucose (2 × 10^5 CFU/ml) and test substance (concentrations 1/64 × MIC − MIC) were added in wells simultaneously. Plates were incubated for 24 h at 37°C. On the other hand, for biofilm dispersal assay, bacterial inoculums (2 × 10^5 CFU/ml) were added, and plates were pre-incubated at 37°C for 24 h to allow bacteria to form biofilms. After that, the medium with planktonic bacteria was removed, wells were rinsed with sterile water, a fresh medium containing emodin concentrations (1/2 × MIC − 4 × MIC) was added and plates were incubated for next 24 h. In both procedures, CV staining was performed after incubation. Plates were rinsed twice with distilled water, 200 µl of 0.1% CV was added in each well and incubated for 15 min at room temperature. After that, plates were rinsed with distilled water, air-dried and 200 µl of absolute ethanol was added to dissolve the remaining CV. Absorbance of dissolved CV was measured at a wavelength of 570 nm on a Microplate reader (Multiskan FC, Thermo Scientific).

Aerobic respiration monitoring

The closed circuit 12-channel Micro-Oxymax respirometer (Columbus Instruments), supplied with an O2 paramagnetic sensor, a CO2 infrared sensor and PC was used to monitor aerobic respiration of planktonic and biofilm forms of S. aureus, with or without emodin treatment. The experiments were performed in Micro-Oxymax light-proof 500 ml bottles, as previously described by Đukanović et al. (2020), with slight modifications (the measurements were performed periodically, at intervals of 4 h). Planktonic form of bacteria was exposed to a sub-inhibitory concentration of emodin (1/2 × MIC), while biofilms were treated with a concentration of 4 × MIC, both during 48 h. Obtained volume of gases was expressed as cumulative consumption and production of O2 and CO2, respectively.

Atomic force microscopy

The effect of the compound on biofilm morphology and structure was analysed by AFM. Samples were prepared by adding 1 ml of bacterial suspension (10^6 CFU/ml) into wells that contained previously placed cover glasses on the bottom. After 24 h of incubation, the culture medium was removed, a fresh medium containing emodin (concentration 4 × MIC) was added and the plates were incubated for 24 h. The next day, the samples were gently rinsed in 1 × PBS (phosphate buffered saline), air-dried and glued to microscopic slides. Afterwards, AFM analysis of the samples was performed with the NTEGRA prima atomic force microscope (NT-MDT). Intermittent-contact AFM mode was applied using NT-MDT NSGO1 silicon, N-type, antimony doped cantilevers with Au reflective coating. The nominal force constant of the cantilevers is 5.1 N/m, whereas the cantilevers driving frequency was around 150 kHz. AFM images were created and analysed with the software Image Analysis 2.2.0 (NT-MDT).

DNA extraction

Genomic DNA from S. aureus strains was isolated using Quick-DNA Fungal/Bactericidal DNA Miniprep Kit (Zymoresearch) according to the manufacturer’s protocol.
Primers and PCR conditions

Specific primers for housekeeping gene *rpO* as well as *icaA*, *icaD*, *srrA* and *srrB* genes are listed in Table 2. The reaction was performed in a total volume of 25 µl of reaction mixture containing components from FastGene TAU PCR Kit (Nippon Genetics) and 1 µl of DNA sample. The amplification was carried out in the conditions described by Kot et al. (2018) in Veriti Thermal cycler 96-well (Applied biosystems).

The amplified PCR products were separated by gel electrophoresis in 1% agarose gels in 0.5 × TE buffer (Tris-EDTA buffer solution) for 1.5 h at 90 V, 300 mA and stained with ethidium bromide. For fragments size amplified with specific primers, FastGene 50 bp DNA Ladder (Nippon Genetics, Europe GmbH) was used. The gels were visualized under UV illumination (2011 MACROVUE, LKB Bromma).

**Extraction of RNA from biofilms, cDNA synthesis and quantitative real-time PCR analysis**

To extract RNA from *S. aureus* biofilms, 1 ml per well of 106 CFU/ml bacterial suspension in MHB with 0.5% glucose was added in six-well plates. After overnight incubation, fresh medium with emodin (4 × MIC) was added, and plates were further incubated for 24 h. Next day, medium with planktonic bacteria was removed, wells were washed with 1 × PBS and total RNA was extracted using trizol reagent (Invitrogen Life Technologies TM). The quality and quantity of RNA were determined spectrophotometrically by BioSpec-nano (Schimadzu Corporation). Reverse transcription for cDNA synthesis was performed in a 20 µl of total volume, containing 2 µg of RNA, using a Maxima HMinus First Strand cDNA Synthesis Kit according to the supplier’s instructions (Thermo Scientific). The reverse transcription was done by incubating the reaction mixture in the Veriti Thermal Cycler (Applied Biosystems), under the following conditions: 10 min at 25°C, 15 min at 50°C and 5 min at 85°C. The expression level of selected genes was quantified by qPCR, conducted on Mastercycler® ep realplex (Eppendorf). Each PCR reaction was prepared in 12.5 µl total volume containing cDNA (30 ng), 500 nM of specific primers (Table 2) and Power SYBR Green PCR Master Mix reagent. Cycling conditions were as follows: 50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 15 s and 60°C for 1 min. The *S. aureus* housekeeping gene (*rpO*) was used as an endogenous control. The relative expression levels of each gene were calculated based on the cycle threshold (Ct) method as described by Tenji et al. (2020).

## Results

### Antibacterial activity

For the examination of antibacterial activity of emodin against *S. aureus* strains, MIC assay was performed. Emodin showed antibacterial effect on all tested strains, with the strongest activity on clinical isolates Gp29 and Gp7 (MIC value 3.125 µg/ml, Table 3).

### Antibiofilm activity

The effect on biofilm formation and preformed biofilms of *S. aureus* was quantified by CV staining of biofilm biomass. Emodin was tested in concentration ranges 1/64 × MIC – MIC and 1/2 × MIC – 4 × MIC respectively, according to previously determined MICs (Table 3). Emodin significantly inhibited biofilm formation of both ATCC strains at all tested concentrations (Figure 1). Biofilm biomass of ATCC 25923 was decreased from 57% to 65% (Figure 1a) while a little bit lower inhibition

**TABLE 3** Antibacterial activity of emodin

<table>
<thead>
<tr>
<th><em>Staphylococcus aureus</em> strains</th>
<th>Minimum inhibitory concentrations µg/ml</th>
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<tbody>
<tr>
<td>MSSA ATCC 25923</td>
<td>25</td>
</tr>
<tr>
<td>Gp41</td>
<td>12.5</td>
</tr>
<tr>
<td>Gp19</td>
<td>6.25</td>
</tr>
<tr>
<td>MRSA ATCC 43300</td>
<td>6.25</td>
</tr>
<tr>
<td>Gp29</td>
<td>3.125</td>
</tr>
<tr>
<td>Gp7</td>
<td>3.125</td>
</tr>
</tbody>
</table>

### Statistical analysis

Data from biofilm formation and dispersal assay and qRT-PCR was analysed by GraphPad Prism 6.01 Software (Software, Inc) using one-way ANOVA. Data obtained in antibiofilm assay are presented as mean value ± standard deviation of three independent experiments done in triplicate, whereas data obtained by qRT-PCR is presented as mean normalized expression ± standard deviation of two independent experiments done in duplicate. To evaluate the statistical significance of differences between mean values of the experiments, Dunnet’s post hoc test was applied. The level of significance was set at *p* < 0.05.
(35%–45%, Figure 1d) was observed on ATCC 43300 strain. On the other hand, emodin demonstrated slightly lower antibiofilm activity on the clinical isolates, except on the Gp29 isolate (inhibition was even 73% at MIC concentration, Figure 1e). The biofilm of the other MRSA isolate (Gp7) was decreased for 25% and 40% at concentrations 1/2 \times \text{MIC} and \text{MIC}, respectively (Figure 1f). Figure 1b and c show that the effect of emodin on MSSA isolates was observed at all tested concentrations for Gp41 (inhibition from 22% to 51%), while on Gp19 it was notably lower (12%–28%) and observed only at 1/2 \times \text{MIC} and \text{MIC}.

Although the effect on inhibition of biofilm formation was expressed on all strains, the effect on mature biofilms was less pronounced. Significant disruption (31%) was noticed on the ATCC 43300 biofilm at 4 \times \text{MIC} concentration, whereas the effect of other concentrations was lower (Figure 2d). Interestingly, the ‘U’ shape was noticed for the effect against this strain. Biofilm biomass of the ATCC 25923 strain remained unchanged after the treatment (Figure 2a). Furthermore, biofilm biomass of the Gp41 strain (MSSA) was slightly reduced with the highest reduction of 14% at 4 \times \text{MIC}, whereas the effect on Gp19 was not detected (Figure 2b,c). The highest decrease in biofilm biomass of the Gp29 and Gp7 (MRSA) strains was 22% at 2 \times \text{MIC} and 23% at 4 \times \text{MIC} concentration, respectively (Figure 2e,f). In addition, at lower tested concentrations the stimulation of the biofilm formation of Gp29 was detected.

**Atomic force microscopy analysis of biofilm**

To investigate the modulatory potential on the surface morphology and structure, emodin was applied in the highest tested concentration (4\text{MIC}), and AFM analyses were performed. Based on the obtained images (Figures 3 and 4), the roughness of biofilm surface was determined.
Changes in biofilm morphology, under the influence of emodin, were observed for the majority of the tested strains. The differences in topography of treated and untreated biofilms of both referent strains especially of ATCC 43300, are evident (Figures 3a and 4a). This observation is also supported with the data extracted from the roughness analysis (Table 4). Sporadically, depressions in biofilm structure, containing residuals of biomass and some cells, could be spotted on treated samples of both referent strains. On the other hand, in AFM images of the treated biofilms of MSSA clinical isolates, especially of the Gp41 strain (Figure 3b,c) less cells were noticed. In addition, this strain biofilm is featured by only a few cell aggregates wrapped with matrix and flat domains between them (Figure 3b). Roughness of Gp41 strain biofilm was more than twice higher compared to control (Table 4). The images of emodin-treated biofilm of the Gp19 strain have different surface morphology including a lower number of cells with more matrix and biomass (Figure 3c), whereas roughness was similar to control. 3D images of treated biofilm of Gp29 strain demonstrated significantly lower number of cell aggregates surrounded with residual biomass (Figure 4b); moreover, the surface roughness of this strain biofilm was lower (Table 4). Contrary to all the other strains, biofilm of the Gp7 isolate treated with emodin preserved the same morphology as its pristine form, with no evident differences in roughness (Figure 4c, Table 4).

**Aerobic respiration measurements**

The potential activity of emodin on aerobic respiration of *S. aureus* in planktonic/biofilm forms was monitored using Micro-Oxymax respirometer by measuring cumulative consumption of O₂ and production of CO₂ at 37°C, performed periodically every 4 h during 48 h (Figures 5 and 6). Concerning respiration in planktonic form (Figure...
5), it was observed that emodin affected bacterial respiration of all the strains after 48 h, but the modality of the detected effect was strain specific. Almost complete inhibition of respiration was spotted in ATCC 25923, Gp41 and Gp29 strain (Figure 5a,b,e) with the highest inhibition of both gasses in Gp41 (85% for O2 and 100% for CO2). Interestingly, only the specific inhibition of CO2 production was noticed in the ATCC 43300, Gp19 and Gp7 strains, amounting to 66%, 71% and 49%, respectively (Figure 5d,c,f).

Furthermore, the effect on aerobic respiration in S. aureus biofilm form was investigated (Figure 6). In the ATCC 25923 strain emodin treatment intensified respiration (the increases of CO2 production and O2 consumption, Figure 6a), whereas in ATCC 43300 only CO2 production was affected (it was decreased for 48%, Figure 6d). Emodin treatment reduced production/consumption of gasses in Gp41 strain: 37% for O2 and 65% for CO2 (Figure 6b). However, aerobic metabolism in the biofilms of Gp19, Gp29 and Gp7 strains was not affected by emodin (Figure 6c,e,f).

**Figure 3** Atomic force microscopy 3D topography images of Staphylococcus aureus MSSA strains biofilms obtained at 10 µm × 10 µm scan areas. The untreated biofilms are presented on the left side, whereas biofilms treated with emodin (4 × MIC) are on the right side.
**TABLE 4**  The effect of emodin on roughness of *Staphylococcus aureus* biofilms

<table>
<thead>
<tr>
<th>Root mean square roughness Rq (nm)</th>
<th>ATCC 25923</th>
<th>ATCC 43300</th>
<th>Gp41</th>
<th>Gp19</th>
<th>Gp29</th>
<th>Gp7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated biofilm</td>
<td>31.8</td>
<td>109.4</td>
<td>105.5</td>
<td>116</td>
<td>182.1</td>
<td>157.8</td>
</tr>
<tr>
<td>Emodin treatment</td>
<td>84.6</td>
<td>66.4</td>
<td>230.7</td>
<td>99.9</td>
<td>117.1</td>
<td>133.1</td>
</tr>
</tbody>
</table>

**FIGURE 4** Atomic force microscopy 3D topography images of *Staphylococcus aureus* MRSA strains biofilms obtained at 10 µm × 10 µm scan areas. The untreated biofilms are presented on the left side, whereas biofilms treated with emodin (4 × MIC) are on the right side.
To confirm the presence of genes of interest (icaA, icaD, srrA and srrB) in reference strains, and to search for their presence in the clinical isolates, PCR was performed using specific primers, and all genes were detected in all tested strains (Figure 7).

**Quantitative real-time PCR**

The effect of emodin on gene expression was quantified by qRT-PCR. The expression of several genes was monitored:

two genes from ica operon (icaA and icaD) and genes from srrAB two-component system. Moreover, the effect of emodin on micro RNA—RNA III was evaluated. Results are expressed as mean normalized expressions with standard deviations (Figure 8). The expression of ica genes in MSSA strains, both ATCC and clinical isolates, was increased in biofilms treated with emodin. As opposed to MSSA strains, the expression of ica genes in all MRSA strains, with the exception of Gp7, was decreased in the presence of emodin. Emodin treatment upregulated srrA and srrB genes of MSSA clinical isolates while in ATCC 25923 strain expression of srrA was almost not affected. In all tested MRSA strains, the effect of emodin...
was consistent and decreased the expression of both genes. Under the influence of emodin, expression of RNA III was also strain specific: The expression of RNA III was reduced in Gp41 and especially in ATCC 25923 and Gp29 strains, whereas in ATCC 43300 and Gp19 strains was increased. However, in the Gp7 isolate level of RNA III remained unchanged.

**DISCUSSION**

Bearing in mind that bacterial resistance has been a global problem for many years, the search for novel antimicrobials and ways to improve the activity of available ones has become intensive. Special attention is directed to natural products, including secondary metabolites of plants and their derivatives, due to their diversity, various biological activities and target specificity (Farha & Brown, 2015). Furthermore, active components of plants generally demonstrate significantly lower toxicity and consequently less pronounced side effects, compared to synthetic therapeutics; additionally, they have not induced resistance (Yang et al., 2018). Emodin, naturally occurring in certain medicinal plants, represents a derivative of anthraquinones with a wide range of biological activities (Cui et al., 2020). There are studies claiming antibacterial activity of emodin...

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**FIGURE 6** Modulation of aerobic respiration in biofilms of *Staphylococcus aureus* ATCC 25923 (a), Gp41 (b), Gp19 (c), ATCC 43300 (d), Gp29 (e) and Gp7 (f) by emodin during 48 h. C–O₂, consumption of O₂ in control; C–CO₂, production of CO₂ in control; T–O₂, consumption of O₂ during emodin treatment; T–CO₂, production of CO₂ during emodin treatment.
(Chalothorn et al., 2019; Yan et al., 2017), but as far as we know there is no enough data concerning its antibiofilm activity and the underlying mechanism. Investigation of antibacterial activity showed that emodin possessed notable antibacterial capacity against all tested strains, with the strongest effect towards MRSA isolates (Gp29 and Gp7), which is in line with previous studies (Chalothorn et al., 2019; Li et al., 2016; Liu et al., 2015; Yan et al., 2017). In addition, Liu et al. (2015) demonstrated that emodin achieves antibacterial activity by damaging the cell wall and membrane of MRSA strains and more importantly, that bacteria have not become resistant to it. A similar mechanism was reported by Liu et al. (2015), who showed that emodin from Polygonum cuspidatum destroyed cell membrane integrity and increased its permeability.

Biofilm is one of the major virulence factors of S. aureus responsible for persistent infections and treatment difficulties. The biofilm formation could be considered as survival strategy against host immunity and applied therapy, during the establishment of the infection (Sacco et al., 2020). Therefore, because this pathogen is also associated with infections that arise from medical device contaminations, prevention of cell attachment and biofilm formation represents a good preventative strategy (Suresh et al., 2019). Taking into account these facts, the next step was to investigate the effect of emodin
on biofilm formation. Results obtained indicated the extraordinary ability of emodin to prevent biofilm formation of all tested strains at all concentrations, with exception of Gp7 strain where that effect was observed only in two the highest concentrations. Similar antibiofilm activity of emodin was reported previously by Yang et al. (2015), who demonstrated that biofilm formation of *Streptococcus suis* was inhibited by emodin at sub-inhibitory concentrations. In addition, it was reported that aloe-emodin inhibited initial adherence and proliferation of *S. aureus* strains (Xiang et al., 2017). These authors suggested that aloe-emodin affected biofilm formation through the inhibition of extracellular protein production and accumulation of PIA. In addition, our results and previously mentioned reports pointed that emodin inhibited biofilm formation at sub-inhibitory concentrations, being in line with Ding et al. (2017) who reported this for many natural products.

Additionally, the effect of emodin on aerobic respiration in planktonic form was monitored for the first time, to examine its mechanism of action and potential targets. The results obtained demonstrated almost complete reduction of respiration in the ATCC 25923, Gp41 and Gp29 strains and less pronounced reduction in all the other tested strains. Interestingly, inhibition of biofilm formation was detected at the same concentrations that reduced aerobic respiration, so the effect on biofilm formation was stronger in the strains with blocked respiration. Similar results have been published for the emodin-reach extract of *Frangula alnus* in our recent paper (Dukanović et al., 2020).

Interest for biofilm investigation and for potential antibiofilm agents is increased in last years, since the highest percentage of total bacterial infections world-wide is related to pathogens able to form biofilms. Biofilm lifestyle provides bacteria additional protection which is reflected in increased resistance and consequently in difficulties in treatment (Penesyan et al., 2020). Since the biofilm is difficult to disrupt, the emodin eradication potential was also examined. Emodin showed potential to disrupt biofilm of all MRSA strains and Gp41 (MSSA), with the strongest potential on the ATCC 43300 biofilm. Previously described antibacterial activity of emodin against MRSA strains, being detected in this work and additionally by several authors (Liu et al., 2015) is in line with the obtained results of biofilm disruption because the MRSA strains were more sensitive to it. Disruption of the ATCC 43300 biofilm was established at all tested concentrations with potentially hormetic dose response. The hormesis is known as a response of cells to different agents which could be either beneficial or detrimental and has specific U-shaped dose dependence (Gambino & Cappitelli, 2016). The most pronounced hormetic response, with notable increase of biofilm biomass at low doses and the highest

**FIGURE 8** Influence of emodin on expression of selected genes in biofilm of *S. aureus* (a) ATCC 25923, (b) Gp41, (c) Gp19, (d) ATCC 43300, (e) Gp29 and (f) Gp7 strains. C, expression of genes in untreated biofilms; E, expression of genes in biofilms treated with emodin; MNE, mean normalized expression. * statistical significance (*p < 0.05)*

![Figure 8](https://academic.oup.com/jambio/article/132/3/1840/6988679)
protection at medium ones, has been observed for the Gp29 strain. It is possible that exposure to low amounts of compounds could trigger adaptive responses of bacteria, to protect them from detrimental activity. However, with the enhancement of detrimental influence, that is, at higher concentrations of emodin, bacterial protective mechanisms were saturated and the reduction of biofilm biomass was observed. Nevertheless, as for typical hormesis, further increment of concentration probably led to the involvement of additional adaptive mechanisms, and consequent minimization of eradicative activity. The fact that antibiofilm activity of emodin, but also of other natural agents (Jodynis-Liebert & Kujawska, 2020; Murakami, 2020) not rarely act in compliance of hormesis phenomenon, potentiate the necessity of careful selection of active doses of antibiofilm agents.

Further on, to confirm the results obtained by CV staining of biofilms, AFM was used. Generally, *S. aureus* is recognizable for the creation of robust, multilayered biofilms, with the clearly observable ability to form three-dimensional structures, shaped by the activity of various enzymes (Archer et al., 2011). *S. aureus* is also known for growth in clusters, both in planktonic and biofilm modes of life, but it also could form large aggregates via PIA formation (Haaber et al., 2012) which could be observed in AFM 3D images of untreated biofilms. The specific architecture of biofilms could be disturbed by stressful environmental conditions, whether the biofilm is to about be disrupted or only the community needs to be protected. Such changes of biofilm morphology and structure were observed in all strains except Gp7. In addition, emodin had impact on biofilm roughness of almost all strains. Interestingly, emodin led to a marked increase in roughness of ATCC 25923 and Gp41 strain. This effect can be attributed to previously reported ability of emodin to interact with cell membrane (Liu et al., 2015). Furthermore, Neethirajan and DiCicco (2014) claims that interaction of antimicrobial agents and the cell membrane modulate cell shape which results in an increase in roughness. However, obtained micrographs demonstrated that emodin managed to disrupt the biofilm into cell aggregates in Gp41 strain and formed depressions in ATCC 43300 biofilms, which is consistent with CV staining results.

To find the new targets that could be of interest for biofilm disruption, the connection between biofilm maintenance and various cellular processes was investigated for years. One of the important characteristics of biofilms is a metabolic heterogeneity that allows tolerance to antimicrobial agents. On the other hand, it is known that activity of antimicrobial agents depends on metabolic status of the cells (Crabbé et al., 2019). According to the fact that *S. aureus* is facultative anaerobe and that aerobic-anaerobic switch certainly influences the structure and activity of biofilm, the effect of emodin on aerobic respiration within biofilms was monitored. That enabled us to search for the correlations between metabolism and biofilm development and possible mechanism of emodin action. Results obtained from the monitoring of biofilms aerobic respiration showed that aerobic metabolism was reduced in ATCC 43300 and Gp41 strains. This result is somewhat expected, given that emodin at these concentrations (4 × MIC) led to the disintegration of the biofilm. It is important to note that even though the biofilm of Gp7 was eradicated by emodin, respiration in treated biofilm remained unchanged. Due to Haaber et al. (2012), *S. aureus* forms cell aggregates, similarly to the biofilm community increased its tolerance to stressful environment and displayed higher metabolic activity. Thus, it could be suggested that emodin caused dispersal of biofilm and the formation of Gp7 strain aggregates, which is reflected in biomass changes but not in metabolic activity.

Since the emodin demonstrated antibiofilm potential, possible pathways of its activity were investigated. *S. aureus* dominantly forms biofilm through PIA synthesis, which is encoded by conserved ica operon, being present in majority of *S. aureus* isolates (Goerke & Wolz, 2010; Haaber et al., 2012). This operon consists of icaA, icaC and icaB genes but icaA and icaD are essential for PIA production (Schilcher & Horswill, 2020) and therefore, the effect of emodin on expression of actually these two genes was monitored. The results obtained by RT-PCR revealed that emodin decreased expression of both ica genes in ATCC 43300 and Gp29 strains. Because the CV staining demonstrated that biofilm biomass of these strains was also decreased, it can be assumed that emodin acts through the inhibition of PIA production. Although biofilm biomass of the Gp41 and Gp7 isolates was reduced, expression of ica genes was upregulated in treated biofilms. Given that biofilm matrix of *S. aureus* beside polysaccharides contains proteins and extracellular DNA (eDNA) (Kannappan et al., 2019), it is possible that emodin affected other matrix components and following research could be focused on them. The expression of srrA and srrB genes, responsible for metabolism control and the aerobic-anaerobic switch, were slightly decreased in ATCC 43300 strain, which is in accordance with reduced aerobic respiration after emodin exposure. These findings reveal that emodin attenuated metabolism of this strain during its biofilm eradication. Contrary, even though biofilm of Gp41 was dispersed, genes of srrAB complex were slightly upregulated, which led to the preposition that cells initiated the metabolic switch to protect themselves. Rudra and Boyd (2020) described that expression of this complex could further repress transcription of RNAIII; the slight reduction was observed in the Gp41 strain. In accordance...
with this, expression of RNAIII was increased in ATCC 43300, since the srrAB complex was decreased. However, emodin in the ATCC 25923 and Gp29 strains significantly downregulated expression of RNAIII, although the upregulation of srrAB complex was not observed. Decreased RNAIII led to increased production of adhesins and ligands, being necessary for the interaction with host cells (Proctor, 2019), which could explain unchanged biofilm biomass of mentioned strains. Novel studies concerning small-noncoding RNAs demonstrate that they have a role in metabolism and biofilm formation and that they could redirect carbon to production of extracellular polymeric matrix (Marincola et al., 2019). On the other hand, reduction of RNAIII allows bacteria to shift from virulent to quiescent form (Tuchscherr et al., 2011) because the RNAIII is part of agr quorum sensing system that control virulence factors of S. aureus.

Obtained results revealed that emodin has great potential as a new antibiotic film agent. Additionally, to find a novel approach in the fight against bacterial biofilms, this paper pointed out the potential importance of the connection between biofilm development and metabolism of bacteria after the treatment with emodin. To the best of our knowledge, this is the first study examining antibiofilm activity of emodin, as well as its mechanism of action and pathways involved.

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CONFLICTS OF INTEREST
The authors have no conflicts of interest to declare.

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