Potential of *Frangula alnus* to contribute to food safety: antibiofilm effect against *Staphylococcus aureus*

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Abstract. Contamination by numerous food-borne pathogens is a major challenge facing the food industry daily. Even though there are many strategies in the fight against contamination, pathogens able to attach to different surfaces and form biofilms are the biggest concern. *Staphylococcus aureus* is a common food-borne pathogen capable of forming biofilms on foods and food contact surfaces. The prevalence of multidrug resistant *S. aureus* is high in raw products, high-protein foods and processed products. Bearing in mind *S. aureus* resistance to numerous antibacterial agents, the aim of this study was to investigate antibiofilm activity of an ethyl-acetate extract of the medicinal plant, *Frangula alnus*, against *S. aureus* ATCC 25923 and *S. aureus* ATCC 43300. It was demonstrated that extract reduced survival of both tested strains by up to 67%. Furthermore, quantification of biofilm biomass showed that extract possesses the extraordinary ability to inhibit biofilm formation of both tested strains (up to 91%). On the other hand, the effect on preformed biofilm was less pronounced and measured only for *S. aureus* ATCC 43300, wherein about 28% of preformed biofilm was eradicated. The results obtained in this study encourage further investigation of *F. alnus* as a novel antibiofilm agent or preservative in the food industry.

1. Introduction

Microbial contamination is the major problem in the food industry that leads to spoilage or contamination of food and, consequently, affects human health. This kind of contamination is unavoidable, since products in any step of processing could be in contact with microorganisms [1]. Pathogenic bacteria could exist and grow on equipment used in the food industry, which allows it to enter food, causing problems in food processing, packaging and consumption.

Despite all preventive measures, good hygiene and disinfection procedures, formation of biofilm by food-borne pathogens is a serious obstacle to safe food. Biofilms are dynamic microbial communities bound to biotic and abiotic surfaces and embedded in self produced extracellular polymeric substances (EPS). The biofilm formation is a complex process that begins with adhesion of planktonic cells to surfaces, followed by their growth and EPS production, resulting in mature biofilms [2]. The biggest issues with biofilms are that they are difficult to eradicate and bacteria in this form are very resistant to adverse events and environments. Furthermore, biofilms could serve as a source for cross-contamination of food, reducing the effectiveness of food processing and quality of food [3].
Among many bacteria present in food and able to form biofilms, *Staphylococcus aureus* is a very important food-borne pathogen and a leading causative of food-borne diseases around the globe. It can easily contaminate high-protein food such as eggs, milk, raw and cooked meat and soybean products [4]. In addition, the ability of *S. aureus* to form biofilms improves its survival on food-contact surfaces and the environment. Furthermore, *S. aureus* in biofilm is chronic source of contamination, since the dispersed cells from the biofilm can continue contaminating food and, thus, interfere with further food chain processes [5]. *S. aureus* has the main advantages of forming multilayered biofilm and being highly resistant to numerous antimicrobial agents.

In order to keep food being contaminated and to extend its expiration date, chemical disinfectants and preservatives are widely used in the food industry. However, constant use of these agents contributes to rapid increase of bacterial resistance and to the agents’ harmful effects [6]. Natural products, such as extracts obtained from medicinal plants, could be safe alternatives to synthetic antimicrobial agents. Since plants have a wide diversity of secondary metabolites, plant extracts possess a number of biological activities that potentially could be exploited [7]. *Frangula alnus* is an interesting, traditionally used medicinal plant with various biological activities, among which its antibiofilm potential is poorly investigated [8].

Taking the above into account, the aim of this study was to investigate the antibiofilm activity of an ethyl-acetate extract of *F. alnus* against the food-borne pathogen *S. aureus*.

2. Methods

In this study two bacterial strains were used: methicillin sensitive (MSSA) *S. aureus* ATCC 25923 and methicillin resistant (MRSA) *S. aureus* ATCC 43300. Firstly, in order to investigate the effect of ethyl-acetate extract of *F. alnus* on bacterial survival, extract was two-fold diluted in Mueller Hinton Broth (MHB) in 96-well microtitre plates and bacterial inoculum (10⁴ CFU/mL) was added. After 24h incubation, optical density was measured at 600nm (OD₆₀₀nm) on a microplate reader (Multiskan FC, Thermo Scientific, Shanghai, China). Tested concentrations of extract were in the range 0.031 mg/mL to 2 mg/mL. Antibiofilm testing included the effect of extract on biofilm formation and on preformed biofilms. The effect was quantified by crystal violet (CV) staining of biofilm biomass as described earlier [9]. Concentrations that were tested were selected based on previously published minimal inhibitory concentrations (MICs) of extract and were in range 1/16×MIC to MIC for biofilm formation and 1/2×MIC to 4×MIC for biofilm disruption [9]. Statistical analysis of data was done by applying GraphPad Prism 6.01 Software (Software, Inc) using one-way ANOVA with Dunnett’s post hoc test. Data of antibiofilm testing are presented as mean value±standard deviation of three independent experiments done in hexaplicate.

3. Results and Discussion

The results obtained by measuring the OD₆₀₀nm showed that *F. alnus* extract had a strong effect on bacterial survival of both tested strains (Figure 1). The highest inhibition for *S. aureus* ATCC 25923 was at concentration 0.5 mg/mL (up to 60% inhibition), while for *S. aureus* ATCC 43300 the highest inhibition was 67% (0.0625 mg/mL). These results are in accordance with some studies that demonstrated antibacterial activity of *F. alnus* extracts at slightly higher concentrations [8, 10]. Differences in reported results could be attributed to extraction procedures, the use of different solvents as well as to growth conditions of plant which could interfere with its chemical composition. In addition, a potentially hormetic dose response was observed in both *S. aureus* strains. The hormesis response is known as a response of cells to different agents that can be either beneficial or detrimental, and which have specific, U-shaped dose dependence [11].
Figure 1. The effect of *F. alnus* extract on *S. aureus* A) ATCC 25923 and B) ATCC 43300 survival. * statistical significance *p* ≤ 0.05

Furthermore, quantification of biofilm biomass revealed that *F. alnus* extract possesses an extraordinary ability to inhibit biofilm formation of both tested *F. alnus* strains in a dose-dependent manner. Biofilm formation of *S. aureus* ATCC 25923 was inhibited by 34.2 to 91.6 % at all tested concentrations, while for *S. aureus* ATCC 43300, inhibition was in range 32.8 to 73.6% (Table 1). It is well known that *F. alnus* is traditionally used because of its high amount of anthraquinones, and Lee et al. (2016) showed the inhibitory activity of this group of secondary metabolites against *S. aureus* biofilms [12]. Thus, the demonstrated antibiofilm activity of ethyl-acetate *F. alnus* extract could be attributed to these compounds. Moreover, it is interesting to note that subinhibitory concentrations of extract were successful in inhibiting biofilm formation, which is in line with a study describing this phenomenon for natural products [13].

### Table 1. The effect of *F. alnus* extract on biofilm formation by *S. aureus* strains

| S. aureus strain | 1/16×MIC | 1/8×MIC | % of inhibition 1/4×MIC | 1/2×MIC | MIC |
|------------------|----------|---------|--------------------------|---------|-----|
| ATCC 25923       | 34.2±0.04| 56±0.13*| 50.7±0.05*                | 84.1±0.14*| 91.6±0.24* |
| ATCC 43300       | 32.8±0.06| 34.3±0.09| 35.5±0.07*                | 71.6±0.09*| 73.6±0.11* |

*Statistical significance *p* ≤ 0.05

On the other hand, the effect on preformed biofilm was less pronounced. Eradication potential was observed for *S. aureus* ATCC 43300, with eradication up to 28% at the MIC (Table 2). In contrast, for *S. aureus* ATCC 25923, a significant increase in biofilm biomass was observed at 2×MIC and 4×MIC (Table 2). Such result could be explained by the fact that some substances could trigger a stress response in biofilms, which leads to the production of extracellular polymeric substances, resulting in increased biomass [14]. Since that extracellular matrix is a means of biofilm resistance, increase of biofilm biomass could be considered as an adaptive response to the *F. alnus* extract [15].

### Table 2. The effect of *F. alnus* extract on preformed biofilms of *S. aureus* strains

| S. aureus strain | 1/2×MIC | % of total biofilm biomass 2×MIC | 4×MIC |
|------------------|---------|----------------------------------|-------|
| ATCC 25923       | 106.8±0.07| 107.2±0.3                        | 135.2±0.5* |
| ATCC 43300       | 85.5±0.17*| 71.7±0.16*                       | 74.9±0.15* |

*Statistical significance *p* ≤ 0.05
4. Conclusion
According to the results obtained in this study, ethyl-acetate extract of *F. alnus* showed strong ability to prevent biofilm formation by both tested *S. aureus* strains. Concerning the results, it is obvious that the inhibitory effect is strain and dose specific. Bearing in mind the problem of food contamination that food industry faces, this biofilm inhibitory activity is of a great importance. However, additional studies are needed in order to apply *F. alnus* as a natural disinfectant or preservative in the food industry and to improve food safety.

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