

Staphylococcus aureus'un Biyofilm Tabakasının Oluşumunda Sumağın Etkisi

ÖZET

Giriş: *S. aureus* hastanelerde çok yaygın olarak izole edilen patojen bakterilerden biridir ve nozokomiyal enfeksiyonların en sık sebebidir. Biyofilm oluşumu ile ilişkili nozokomiyal stafilokok yabancı cisim enfeksiyonları ciddi tehdittir ve yeni tedavi ile önleyici stratejiler gerektirir. İntravenöz kateterlerin implantasyonu, prostetik implantların cerrahi implantasyonu enfeksiyon riski taşır. Biyofilmlerin tüm bu etkilerini önleyebilmek için sumağın *S. aureus* biyofilm oluşumu üzerinde olası antibakteriyel etkilerini gözlemlemek için bu çalışma tasarlandı.

Gereç ve Yöntem: Sumağın çeşitli konsantrasyonlarının 13 *Staphylococcus aureus* suşunun biyofilm oluşumuna etkisi mikroelisa yöntemiyle test edildi.

Bulgular: Dört metisilin dirençli *Staphylococcus aureus* (MRSA) ve dokuz metisilin duyarlı *Staphylococcus aureus*'ta (MSSA) sumağın değişik konsantrasyonları (0.1, 0.2, 0.5 ve 1.0 µl/ml) arasında anlamlı farklılıklar gözlemlendi ($p < 0.05$). Bakterilerin tümünde stafilokok enfeksiyonlarında önemli bir virülans faktörü olan slime üretiminde dozla ilişkili azalma gözlemlendi.

Sonuç: Çalışmamızda, sumağın 0.1, 0.2, 0.5 ve 1.0 µl/ml konsantrasyonlarında 13 suşun biyofilm üretimi sırasıyla 17%, 22%, 28% ve 48% azaldı. Bitkisel bir ürün olan sumağın özellikle MRSA olmak üzere stafilokok enfeksiyonlarında önemli bir virülans faktörü olan biyofilm üretimini azaltabildiği sonucu çıkarılabilir.

Anahtar Kelimeler: *Staphylococcus aureus*; Biyofilm oluşumu; *Rhus coriaria*; Yabancı cisim enfeksiyonları.

Effects of Fermented Sumach on The Formation of Slime Layer of *Staphylococcus aureus*

ABSTRACT

Objectives: *Staphylococcus aureus* (*S. aureus*) is one of the most commonly isolated bacterial pathogens in hospitals, and the most frequent cause of nosocomial infections. Nosocomial staphylococcal foreign-body infections related to biofilm formation are a serious threat, demanding new therapeutic and preventive strategies. Implantation of intravenous catheters, surgical implantation of prosthetic implants carries risk of infection. In order to prevent all these effects of biofilms a study was designed to observe the possible antibacterial effect of sumach (*Rhus coriaria*) on the biofilm formation of *S. aureus*.

Material and Methods: The influence of varying concentrations of sumach on the formation of biofilms by 13 strains of *Staphylococcus aureus* was tested by microelisa assay.

Results: The significant differences between varying concentrations of sumach (0.1, 0.2, 0.5 and 1.0 µl/ml) were observed in four methicillin resistant *Staphylococcus aureus* (MRSA)

47 and nine methicillin sensitive *Staphylococcus aureus* (MSSA) ($p < 0.05$). In bacteria, a dose-
48 related decrease in the formation of slime which is a major virulence factor of staphylococcal
49 infections was observed.

50 **Conclusion:** In our study, using 0.1, 0.2, 0.5 and 1.0 $\mu\text{l/ml}$ of sumach, thirteen strains lost,
51 respectively, 17%, 22%, 28% and 48% of their capacity to produce biofilms. Sumach which is
52 a herbal product can decrease formation of biofilm which is a major virulence factor in
53 staphylococcal infections.

54 **Keywords:** *Staphylococcus aureus*; Biofilm formation; *Rhus coriaria*; Indwelling device-
55 associated infections.

56

57 **Introduction**

58 The increasing numbers of multidrug-resistant Gram-positive pathogens have
59 generated worldwide concern in the medical community. The emergence and spread of the
60 methicillin resistant *S.aureus* (MRSA) has been shown to be associated with both hospital- and
61 community-acquired infections. Effective treatment options for these infections are limited
62 and the situation may become more severe soon. For these reasons, a proactive management
63 of MRSA in healthcare facilities is needed (1, 2). The use of different types of antibiotics
64 over the years has led to the emergence of multi-resistant MRSA strains (3). Although the
65 types and severity of diseases produced by the opportunistic pathogen, *S.aureus* vary, it was
66 reported to be a frequent cause of infections associated with indwelling medical devices (e.g.,
67 catheters and artificial heart valves) (4).

68 In a biofilm, bacteria are well protected from the host immune defense. An increase in
69 antibiotic resistance is the consequence (5, 6, 7) even high local concentrations of antibiotics
70 do not completely eradicate bacteria in biofilms (8, 6).

71 The increasing occurrence, particularly in hospitals, of *S. aureus*' resistance including
72 methicillin and a wide range of antimicrobial agents like all kinds of β -lactams, has made
73 therapy more difficult (9, 10, 11, 12). Although strategies have been proposed in an attempt
74 to control the spread (13), the search for new ways to treat MRSA infections stimulates the
75 investigation of natural compounds, as an alternative treatment of these infections.

76 Indwelling device-associated infections commonly involve the formation of a bacterial
77 biofilm on an uncoated plastic surface or on a plastic surface coated with host proteins (4).

78 *Rhus coriaria* (Sumach) and some other species of *Rhus* brought powdered leaves and
79 fruits that they have antibacterial properties have also been reported by other researchers (14,
80 15). Sumach is rich in water-soluble tannins, and the antimicrobial activity of tannins is well
81 documented (16). Nasar-Abbas and Halkman (2004) have demonstrated that not only the
82 organic acids but also the other substances in water-extracted sumach were found to be
83 effective antimicrobial agents (17). It is generally believed that the fully protonated species of
84 organic acids can diffuse into the bacterial cells, and cause cell death (18, 19, 20). The
85 fermented sumach is widely used as a salad dressing in Southern East provinces of Turkey.

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88 **Materials And Method**

89 **The Bacteria.** Thirteen *S. aureus* isolates which had been recruited from the samples of
90 patients who have visited the microbiology laboratory of the hospital of Abant Izzet Baysal
91 University, The Faculty of Medicine, Bolu, Turkey. Four of these strains were found to be
92 methicillin resistant *Staphylococcus aureus* (MRSA) and nine of them were reported as
93 methicillin sensitive *Staphylococcus aureus* (MSSA), in a previous study were inoculated
94 into the blood agar and grown at 37° C for a period of 24 hours. Upon following the growth,
95 the relevant isolates were treated with the fermented sumach which was put in the tryptic soy

96 broth (TSB) (Merck TM) at 24 hours and 37° C in an incubator with respect to the controls of
97 the isolates, which were studied in non-fermented sumach added TSB for 24 hours and 37° C
98 in an incubator.

99 **The Fermented Sumach.** The fermented sumach was obtained from a local vendor in
100 Gaziantep, Turkey. It was prepared by grinding with up to 20% salt and left for fermentation.

101 **The Experimentation.** The treatment including four different concentrations of the fermented
102 sumach were added to each microtiter plate in microelisa reader instrument (Thermo
103 InstrumentsTM), containing TSB and analyzed separately. The concentrations of the
104 fermented sumach were 0.1, 0.2, 0.5 and 1 µl/mL. In all microtiter plates, TSB was used and
105 the process was repeated in triplicates. The isolates were inoculated to cuvettes (LP Italiana
106 SPA TM) which contained the treated and non-treated groups.

107 **The Qualitative Determination of Slime.**

108 **(i) Congo red Agar method (CRA):** In order to screen out the biofilm formation by *S.*
109 *aureus*, the bacteria were grown on Congo red agar (Merck TM) as described by Freeman et al.
110 (1989) (21). The colony morphology was examined after 24 h at 37°C. Positive result was
111 indicated by black colonies.

112 **(ii) Tube method.** The case study was verified by an assay, in which the biofilm formation
113 by bacteria was additionally detected by another method described by Christensen et al.
114 (1985) by overnight cultivation of *S. aureus*, inoculated in polystyrene test tube which
115 contained TSB as an alternative (22). The biofilms formed on the walls of polystyrene test
116 tube were washed twice with phosphate-buffered saline (PBS) to remove the planktonic cells.
117 Then, the cells were stained with saphranin for 1 hour. After discarding saphranin,
118 polystyrene test tube was washed twice with PBS and followed the air drying of the
119 polystyrene test tube. Slime production was judged to have occurred if a visible film lined the
120 walls of the tube (22). The adherent bacterial films were measured spectrophotometrically at
121 540 nm in a microplate reader (Thermo Instruments TM). This process was repeated with 0.1,
122 0.2, 0.5 and 1 µl/mL concentrations of sumach treated TSB to determine the effects of sumach
123 on slime production of isolates. The studies were repeated in triplicates.

124 **The Quantitative Determination of Slime.**

125 **(i) Spectrophotometric method:** The different concentrations of sumach were, mixed with
126 TSB and non-treated TSB were used for controls. The optical density (OD) value of the
127 inoculum was adjusted to approximately 0.600 by a spectrophotometer (Hitachi TM). 200 µl
128 of bacterial suspension were inoculated into 96-well flat-bottomed sterile polystyrene
129 microplate (LP Italiana SPA TM) which contained TSB. Some wells were left free of
130 fermented sumach as controls and incubated for 24 h at 37°C. The biofilms formed on the
131 plates were washed twice with phosphate-buffered saline (PBS) to remove the planktonic
132 cells. Then, the cells were stained with saphranin for 1 hour. After discarding saphranin, the
133 microplate was washed twice with PBS and followed the air drying of the wells. The
134 adherent bacterial films were measured spectrophotometrically at 540 nm in a microplate
135 reader (Thermo Instruments TM). This process was repeated with 0.1, 0.2, 0.5 and 1 µl/mL
136 concentrations of sumach treated TSB to determine the effects of sumach on slime production
137 of isolates. The studies were repeated in triplicates.

138 **The Determination of The Slime Index (SI).** Following a period of 24 hours' incubation of
139 isolates which are treated with the different concentrations of the fermented sumach, the
140 growth of *S. aureus* were confirmed with the microelisa reader instrument (Thermo
141 Instruments TM). The O.D. value of the biofilm was corresponding with the value in O.D. of
142 bacterial growth determined spectrophotometrically, before the aspiration of the culture in
143 order to compensate the partial inhibition in growth caused by the fermented sumach and this
144 was termed as the slime index (SI). The result was expressed as percentage relative to the

145 control without fermented sumach. For this purpose the following formula was applied: $SI =$
 146 $100 \times (\text{mean density of biofilm with supplement}/\text{mean growth with treatment})/(\text{mean density}$
 147 $\text{of biofilm without treatment}/\text{mean growth without treatment})$ (Pérez-Giraldo C. et al., 1997)
 148 (23).

149 **Statistical analysis.** The Friedman test was used to detect the existence of differences in
 150 growth and biofilm formation among the different groups. The significant level was set for p
 151 $< 0,05$ in the evaluation of Friedman test results. Where significant differences existed,
 152 comparison between the concentrations of sumach was carried out by the two related sample
 153 test (Wilcoxon test). Bonferroni correction was made in the evaluation of p values which were
 154 obtained from Wilcoxon test. The significant level was set for $p < 0,017$ in the evaluation of
 155 Wilcoxon test results.

156 157 **Result**

158 The 13 strains of *S. aureus* included in this study were found to be biofilm-producing.
 159 Strains which were not treated with sumach produced a slime layer of which O.D. value
 160 ranging from 0.074 to 0.389. A total of 13 strains gave an O.D. of > 0.100 . Strains which
 161 were treated with sumach decrease a biofilm of which O.D. value ranging from 0.082 to
 162 0.070. The results of growth and biofilm formation in the presence of different concentrations
 163 of sumach determined by spectrophotometrical assays are presented in the Table 1.

164 It was found that there were significant differences in growth between the
 165 concentrations of 0.1 and 1.0 $\mu\text{l/ml}$ (Table 2). In addition, there were significant differences
 166 in biofilm formation of MSSA and MRSA between concentrations of sumach. Probably the
 167 decrease in the O.D. of the biofilms was directly proportional to the fermented sumach
 168 concentration. The fermented sumach, served in four different concentrations showed the
 169 same effect on the biofilm formation and the growth of MSSA and MRSA ($p < 0.05$).

170 The reduction in SI and slime which is a major virulence factor of staphylococcal
 171 infections proved to be statistically significant at four concentrations of sumach. At four
 172 concentrations sumach decreased the biofilm formation of 13 strains and reduced the biofilm
 173 formation by 48% at a concentration of 1.0 $\mu\text{l/ml}$ (Table 1). The mean percentage of biofilm
 174 of all the strains relative to the control, with a concentration of 0.1, 0.2, 0.5 $\mu\text{l/ml}$ and 1.0
 175 $\mu\text{l/ml}$ of sumach, was 77.42 ± 2.67 , 71.18 ± 2.52 , 63.25 ± 2.18 ($p < 0.05$) and 52.51 ± 1.98 (p
 176 < 0.05), respectively (Table 1). The fermented sumach demonstrated a dose-dependent slime
 177 reducing activity (Table 1 and 2). But SI indicates that there is no significant decrease in the
 178 biofilm between the concentrations of 0.1 $\mu\text{l/ml}$ and 0.2 $\mu\text{l/ml}$ ($p < 0.017$), and between the
 179 concentrations of 0.2 $\mu\text{l/ml}$ and 0.5 $\mu\text{l/ml}$ (Table 2). So, Biofilm inhibition effects of sumach
 180 are the same at the concentrations of both 0.1 $\mu\text{l/ml}$ and 0.2 $\mu\text{l/ml}$ ($p < 0.017$) and the same at
 181 the concentrations of both 0.2 $\mu\text{l/ml}$ and 0.5 $\mu\text{l/ml}$ (Table 2) but are less than the inhibition
 182 effect of the concentration of 1.0 $\mu\text{l/ml}$ (Table 1). The most effective concentration is 1.0
 183 $\mu\text{l/ml}$ in biofilm inhibition (Table 1).

184 185 **Table 1. The Friedman test results which show the effects of different concentrations of sumach on the**
 186 **growth and biofilm formation of 13 isolates**

	Sumach (mean \pm std.deviation)				df	N	p
	0.1 $\mu\text{l/ml}$	0.2 $\mu\text{l/ml}$	0.5 $\mu\text{l/ml}$	1.0 $\mu\text{l/ml}$			
SI	83.40 \pm 28.75	78.46 \pm 3.03	71.91 \pm 2.83	51.92 \pm 2.45	3	13	.000 *
Slime	77.42 \pm 2.67	71.18 \pm 2.52	63.25 \pm 2.18	52.51 \pm 1.98	3	13	.000 *
Growth	96.64 \pm 3.27	94.69 \pm 3.59	90.88 \pm 2.93	1.08 \pm 4.36	3	13	.009 *

187 * $p < 0.05$

188 **Table 2. The Wilcoxon test results which show the effects of different concentrations of sumach on the**
 189 **growth and biofilm formation of 13 isolates**

		Concentrations of sumach intervals ($\mu\text{l/ml}$)					
		0.2 $\mu\text{l/ml}$ – 0.1 $\mu\text{l/ml}$	0.5 $\mu\text{l/ml}$ – 0.1 $\mu\text{l/ml}$	1.0 $\mu\text{l/ml}$ – 0.1 $\mu\text{l/ml}$	0.5 $\mu\text{l/ml}$ – 0.2 $\mu\text{l/ml}$	1.0 $\mu\text{l/ml}$ – 0.2 $\mu\text{l/ml}$	1.0 $\mu\text{l/ml}$ – 0.5 $\mu\text{l/ml}$
p	SI	.099	.010 *	.001 *	.059	.001 *	.002 *
	Slime	.005 *	.001 *	.001 *	.002 *	.001 *	.001 *
	Growth	.701	.152	.011 *	.600	.004 *	.005 *

* $p < 0.017$

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192 Discussion

193 Methicilin resistant *S.aureus* (MRSA) has been shown to be associated with both
 194 hospital- and community-acquired infections (1, 2). Seyit Ali Büyüktuna et al. (2010) have
 195 demonstrated that one of the pathogen of nosocomial infections was Staphylococcus spp.
 196 (16.7%) in intensive care unit (24). The choice of drugs, to be used against MRSA, is
 197 shrinking day by day; as susceptibility of MRSA to drugs is decreasing by target site
 198 alteration, enzyme modification and permeability changes (25).

199 Studies have been made to decrease adherence of coagulase negative *Staphylococcus*
 200 (CoNS) to catheters by coating them with antiseptics and silver, or by salicylic acid and some
 201 other nonsteroidal anti-inflammatory drugs (26, 27).

202 Several studies have been made to manage with microbial biofilm on the biomaterials,
 203 including the incorporation of antibiotic and non-antibiotic agents (e.g. usnic acid,
 204 epigallocatechin-gallate, ovotransferin, protamine sulfate, surfactin) into biomaterials (28,
 205 29). The incorporation of antibiotics on catheters seems to be inappropriate to prevent biofilm
 206 formation, in contrast to non-antibiotic agents, it can lead to bacterial resistance to
 207 antimicrobial agents (28). One study showed that the adhesion and formation of the *S.*
 208 *epidermidis* biofilm on the PCV Nelaton and Thorax catheters had been inhibited by EDTA at
 209 low concentrations (between 1–2 mmol/l) (30).

210 In our data, using 0.1, 0.2, 0.5 and 1.0 $\mu\text{l/ml}$ of sumach, thirteen strains lost 17%, 22%,
 211 28% and 48% of their capacity to produce biofilms. Perez Giraldo et al. (24) and Marek Juda
 212 et al. (30) studied the effect of EDTA on the formation of biofilm by *S. epidermidis*.
 213 According to data of Perez Giraldo et al. (24) with the highest concentrations (0.25–8 mg/mL)
 214 the O.D. of the biofilms diminished and using 1 mg/mL, eight strains lost 75% of their
 215 capacity to produce biofilms. According to Marek Juda et al. (30), EDTA inhibited adhesion
 216 and biofilm formation by the *S. epidermidis* isolates on biomaterials at concentrations of 1.0–
 217 2.0 mmol/l.

218 Our results show that sumach decreases growth-independent formation of biofilm
 219 which is a major virulence factor of staphylococcal infections. For this reason, sumach may
 220 be an effective alternative for preventing indwelling prosthetic infections by *S. aureus*. This
 221 study has demonstrated that the higher dose of sumach, the lower the formation of the
 222 biofilms. In the presence of 0.1 $\mu\text{l/ml}$ of sumach or more than this concentration, the results
 223 were statistically significant. The sumach which include four different concentrations had the
 224 same effect on biofilm formation and growth of MSSA and MRSA.

225 It would be appropriate to confirm these results by animal experiments. According to
 226 this possible confirmation, applications of sumach can be researched. Sumach may be
 227 administered by direct instillation, orally. However, it may be possible by local application to
 228 obtain useful concentrations to prevent the formation of biofilms and adherence of *S. aureus*.
 229 This herbal product may be incorporated to indwelling devices for preventing adhesion of *S.*
 230 *aureus* to medical device. Its hard to put out infected device, by this way this can be prevented
 231 by sumach. Indwelling device associated infections even MRSA infections may be prevented

232 without using antibiotics or other chemicals which can provide resistance of bacteria. If these
233 results would be confirmed by animal and clinical experiments, freeze dried tablets which
234 contain ingredients of sumach may be produced by drug companies too.

235 In conclusion, our results suggest that sumach may prevent the formation of biofilms
236 and adherence of *S. aureus*. When incurable indwelling device associated infections arise by
237 *S. aureus*, sumach can be an alternative treatment option if this will be confirmed by animal
238 and clinical experiments. We consider that it would be appropriate to carry out animal and
239 clinical studies to confirm this.
240

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