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Rosmarinic acid inhibits biofilm formation of *Streptococcus mutans*

Emerging antimicrobial resistance and adverse effects associated with antimicrobial over use necessitate new antimicrobial therapeutics with natural compounds considered as attractive alternatives to synthetic drugs. Rosmarinic acid is abundant in medicinal plants. The aim of this study was to elucidate its possible role as an inhibitor of *Streptococcus mutans* biofilm growth. The amount of biofilm formed by *S. mutans* bacteria was estimated using colorimetric method and optical profilometry. In this study, rosmarinic acid showed significant inhibitory activity at 5 mg/mL concentration on *S. mutans* biofilm formationin 1 % sucrose containing medium. Considering the broad antimicrobial and antibiofilm spectrum of activity, rosmarinic acid can be used as an antimicrobial agent along with a number of medicinal plants containing rosmarinic acid as a dominant compound. However, rosmarinic acid can serve as a basis for the development of antimicrobial and therapeutic and prophylactic drugs used in dental practice.

Keywords: colorimetric method, optical profilometry, biofilms, *Streptococcus mutans*.

Introduction

Rosmarinic acid (RA) is an organic compound belonging to the group of phenolic acids. RA is probably one of the most well-known secondary metabolites of plants. It is most often and in large quantities found in plants of the *Lamiaceae* family: rosemary (*Rosmarinus officinalis*), sage (*Salvia officinalis*), basil (*Ocimum basilicum*), lemon balm (*Melissa officinalis*) [1]. RA has a wide range of beneficial properties and is currently being actively studied in scientific and medical research. The chemical structure of RA is shown in Figure 1.

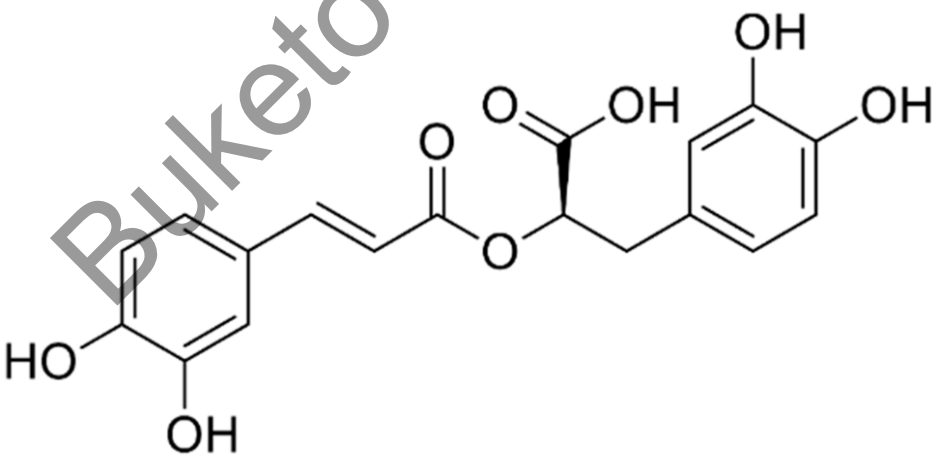

Figure 1. Chemical structure of rosmarinic acid

RA ($C_{18}H_{16}O_8$) contains several key chemical groups that determine its properties and functions. Phenolic groups in plants play an important role in their biological activity and have a variety of properties, thus the two phenolic rings present in RA give it antimicrobial and antioxidant properties [2]. In addition to phenolic groups, RK contains carboxyl, hydroxyl and ether groups that affect solubility and participate in the formation of hydrogen bonds [3]. RA is used for application in various industries such as cosmetology, medicine, food industry, pharmaceutical, and agricultural industry [4].

Dental caries is currently one of the most common dental problems worldwide and is a serious problem for the population, especially for children. *Streptococcus mutans* is a facultative aerobic gram-positive bacterium and an important cariogenic pathogen. This bacterium inhabits the human oral cavity, causing dental plaque and dental caries [5]. The main virulence factors of *S. mutans* are the ability to form biofilms attached to the tooth surface, the ability to produce organic acids (acidity), and viability under low pH conditions (acidity) [6]. *S. mutans* has the ability to adhere to tooth enamel, forming the initial layers of biofilm (plaque). These bacteria use specific adhesive molecules such as adhesins and exopolysaccharides to attach to the tooth surface [7].

RA exhibits antimicrobial properties against gram-positive strains: *Staphylococcus aureus*, methicillin-resistant *Staphylococcus aureus*, *Bacillus cereus*, *Enterococcus faecalis*, *Listeria monocytogenes*; gram-negative strains: *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella* spp*., Klebsiela pneumoniae* [8, 9]. However, the literature does not contain studies on the effect of RA on the formation of biofilm caused by the growth of *S. mutans* bacteria. In this regard, it is relevant to study the effect of RA on the formation of *S. mutans* biofilms. Given the high antimicrobial activity, RA has the potential for use in the development of new dental and prophylactic agents.

Experimental

Plant material. Wild plant of the flora of Kazakhstan *Salvia stepposa* Des. -Schost (synonym *Salvia dumetorum*) was collected during expeditions in the Karaganda region, Republic of Kazakhstan, collection coordinates (N 49°88898’; E 73°15569’) in the budding and flowering phase in July-August 2023.

Isolation of RA. Rosmarinic acid was isolated from *Salvia stepposa* according to the methods described in the literature [10, 11].

Biofilm formation, processing and analysis by colorimetric analysis. Experimental data were obtained using the method described in the literature [12]. *Streptococcus mutans* strain UA159 (ATCC 700610) was cultured in Todd-Hewitt (TH) broth under anaerobic conditions (95 % N_2 and 5 % CO_2) at 37 °C for 18 h before the experiments. 1000 mg of rosmarinic acid was dissolved in 10 mL of pure dimethyl sulfoxide (DMSO) to obtain stock solutions with a concentration of 100 mg/mL. The prepared stock solution of RA was stored at –35 °C until use.

At the beginning of the experiments, 24-well flat-bottomed polystyrene cell culture plates were filled with TH containing 1 % sucrose, and then RA solution was added to the corresponding wells at final concentrations of 1 mg/mL, 2.5 mg/mL, 5 mg/mL, 7.5 mg/mL, and 10 mg/mL. Three RA concentrations were selected from this range for the treatment of *S. mutans* bacteria. DMSO solvent was added to the corresponding wells at final concentrations of 1 %, 2.5 %, 5 %, 7.5 %, and 10 % (v/v).

Before each experiment, the optical density (OD) of the bacterial culture was adjusted to 0.2 at 595 nm using a BioTek Synergy HTX microplate reader. *S. mutans* bacteria were then added to the wells of the plate containing RA at a final dilution of 1:100, and all plates were incubated anaerobically (95 % N_2 and 5 % CO_2) at 37 °C for 24 h. In the experiments, wells of the plate without bacterial cells were used as blank controls, while untreated bacteria without sucrose served only as an internal control for the experiments and were not included in the calculations.

After 24 h of incubation, TH was removed from the plates, the wells were washed with distilled water to remove loosely adherent cells, and then the adherent bacteria were fixed with 95 % ethanol. The fixed and air-dried *S. mutans* biofilm in the wells of the plate was stained with 0.01 % crystal violet for 15 min.

The bound dye was extracted with 33 % acetic acid for 30 min. Afterwards, 200 µl of the extracted dye solution from each well was transferred to the corresponding wells of an optically clear flat-bottomed 96-well microplate. The OD of the samples was measured at 595 nm using a BioTek Synergy HTX microplate spectrophotometer. Background staining was corrected by subtracting the amount of staining in the empty wells (Fig. 2).

The percentage inhibition of biofilm formation was calculated using the OD values (%) according to the equation:

$$\%\ \text{of inhibition} = \frac{x(\text{control}) - x(\text{treatment})}{x(\text{control})} \cdot 100\%$$

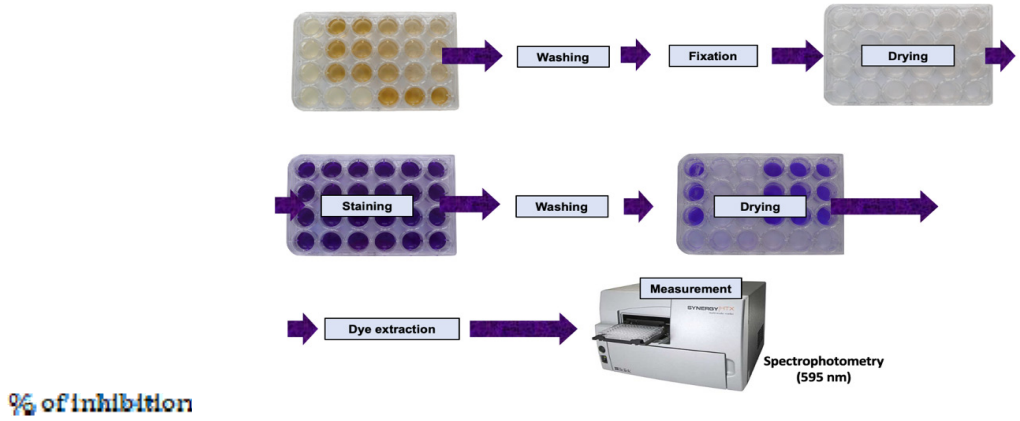

Figure 2. Colorimetric assay [13]

Statistical analysis. Data were analyzed using SPSS version 23.0 (IBM Corp., Armonk, NY, USA). Differences between the control (untreated) and treatment groups were assessed using one-way analysis of variance followed by the least significant difference post hoc test for multiple comparisons. Data are presented as mean ± standard error. $P< 0.05$ was considered to indicate a statistically significant difference.

Results and Discussion

Despite the widely studied antimicrobial activity of RA, no data on its effectiveness against *S. mutans* have been found. However, other phenolic acids or their derivatives showed antimicrobial activity against biofilm formation of *S. mutans*. Scientists have found that rosemary extract (*Rosmarinus officinalis*) has an antimicrobial effect on *S. mutans*. Studies show that rosemary extract can effectively reduce the total protein level in *S. mutans* biofilms, achieving a reduction of approximately 32 % [14]. Studies have been published on carnosic acid and carnosol, which as RA, belong to the class of phenolic plant metabolites, confirming the activity of these compounds against *S. mutans* at concentrations of 40 μg/mL and 75 μg/mL, respectively [15]. Other authors have found that the flavonoids quercetin and kaempferol also reduce *S. mutans* biofilm formation compared to the control [16]. Caffeic acid derivate such as caffeic acid phenethyl ester (CAPE) showed a good inhibitory effect on the biofilm-forming and cariogenic abilities of *S. mutans*. CAPE (0.04 mg/mL) inhibited biofilm formation by at least 50 %, and at 0.08 mg/mLCAPE inhibited biofilm formation by more than 90 %. Additionally, CAPE can inhibit crucial virulence factors of *S. mutans* related to its cariogenic potential, such as acid production, acid tolerance, and the synthesis of extracellular polysaccharides, without compromising bacterial viability at lower concentrations [17].

Evaluation of the efficacy of RA in inhibiting *S. mutans* biofilm formation using a colorimetric assay showed the ability to significantly inhibit *S. mutans* biofilm formation in a dose-dependent manner on the polystyrene surface of 24-well cell culture plates. Treatment with RA at a concentration of 2.5 mg/mL resulted in only a slight reduction in biofilm formation at the bottom of wells in 24-well cell culture plates (Fig. 3).

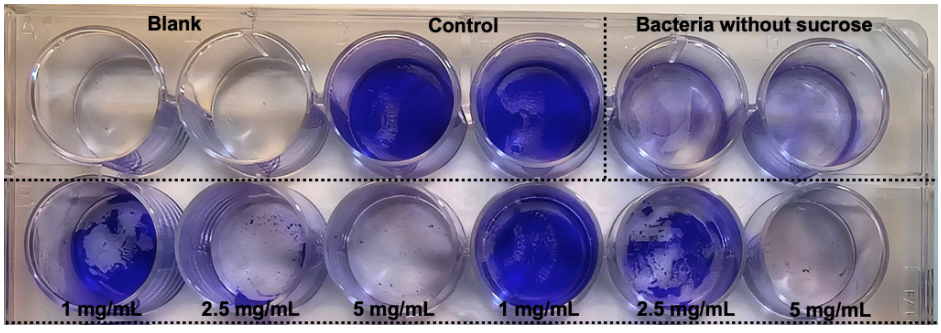

Figure 3. *S. mutans* biofilm stained with 0.01 % crystal violet solution after 24 h of incubation in the presence of RA

DMSO significantly reduced the biofilm formation of *S. mutans*, except for the DMSO concentration of 1 % (Fig. 4). However, the inhibitory activity of RA at a concentration of 5 mg/mL significantly exceeded the experimental results compared to the corresponding DMSO concentrations.

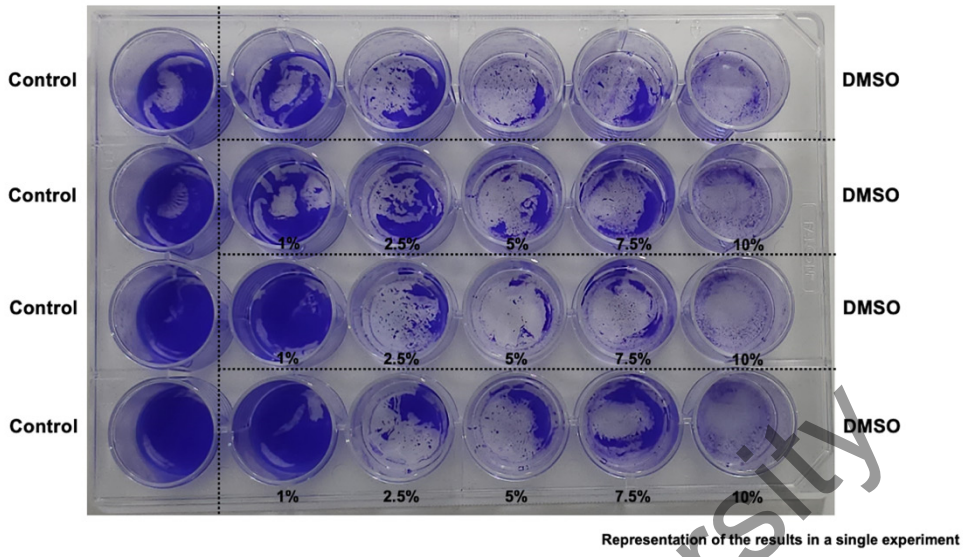

Figure 4. *S. mutans* biofilm stained with 0.01 % crystal violet solution after 24 h of incubation in the presence of dimethyl sulfoxide (DMSO)

As shown in Figure 5, RA at a concentration of 1 mg/mL does not reduce the formation of *S. mutans* biofilm. However, with an increase in the concentration from 2.5 mg/mL to 5 mg/mL RA, positive dynamics are observed in reducing the formation of *S. mutans* biofilm by 54 % (*p < 0.05 compared to the control) and 90 % (**p < 0.05 compared to DMSO), respectively.

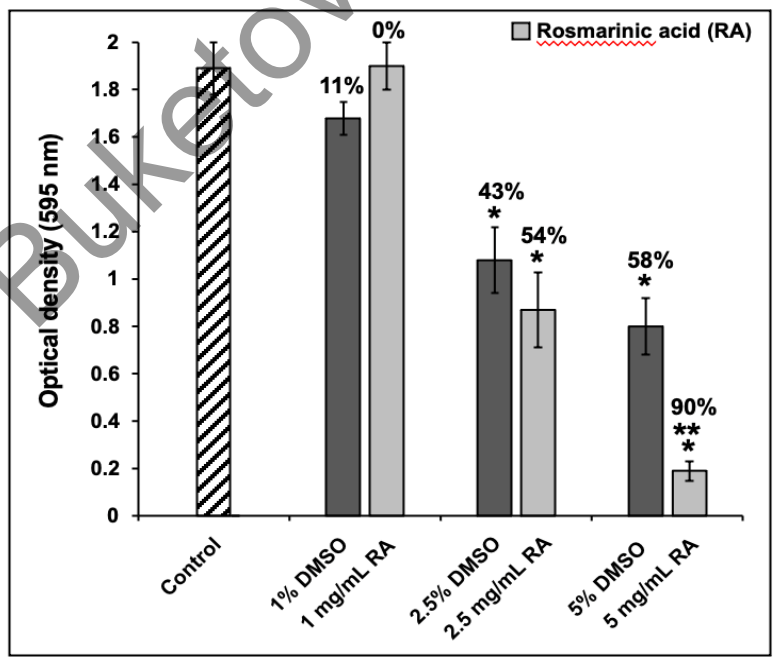

Figure 5. Optical density of *S. mutans* biofilm after 24 h of incubation in the presence of RA (*p < 0.05 compared to control; **p < 0.05 compared to DMSO). Values are mean ± standard error of four independent experiments (n=4–18); One-way ANOVA, LSD Post Hoc test (SPSS software, version 23.0); Percentages indicate the inhibitory effect of extracts compared to the control (untreated bacteria).

Thus, RA exhibits inhibitory activity against *S. mutans* biofilm formation in a medium containing 1 % sucrose (which is the main inducer of biofilm formation for *S. mutans* bacteria). The solvent DMSO also reduces *S. mutans* biofilm formation in a concentration-dependent manner in a medium containing 1 % sucrose, but the inhibitory activity of RA against *S. mutans* biofilm formation is slightly higher compared to DMSO.

Conclusion

RA is an important secondary metabolite of plants, which finds its wide application due to its diverse spectrum of biological activity. *S. mutans* is a major cariogenic pathogen that contributes to the occurrence of many oral diseases. The best treatment option is the selective exclusion of dental caries. Anti-biofilm agents can inhibit the growth of *S. mutans* in the microareas of teeth, dental restorations or implant-supported prostheses. However, currently oral antimicrobial agents are mainly used as broad-spectrum bactericides, and they poorly regulate the production of both biofilms and virulence factors. In this regard, in this study, the potential of RA, which is an easily renewable metabolite obtained from plants, in inhibiting the biofilm formation of *S. mutans* was investigated.

As a result of the experiment, it was revealed for the first time that RA has a significant biological effect and can protect teeth from damage caused by *S. mutans*. It was found that RA exhibits the greatest suppressive effect on the formation of *S.mutans* biofilm in a medium containing 1 % sucrose at a concentration of 5 mg/mL. The results of the study can be used to develop new therapeutic and prophylactic dental products.

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Streptococcus mutans биоқабықшасының түзілуін тежеуші Розмарин қышқылы

Антибиотиктерді шамадан тыс қолданумен байланысты дамып келе жатқан микробқақарсы төзімділік пен жанама әсерлер, тартымды балама ретінде қарастырылатын табиғи қосылыстары бар жаңа микробқақарсы терапияның қажеттілігін туғызды. Розмарин қышқылы дәрілік өсімдіктерде көп кездеседі. Зерттеудің мақсаты розмарин қышқылының *S. mutans* биоқабықшасының түзілуін тежеуші ретіндегі ықтимал рөлін анықтау. *S. mutans* бактериялары түзетін биоқабықша мөлшері, колориметриялық әдіс пен оптикалық профилометрия көмегімен бағаланды. Бұл зерттеуде розмарин қышқылы 5 мг/мл концентрацияда, құрамында 1 % сахароза бар ортада *S. mutans* биоқабықшасының түзілуін айтарлықтай тежеу белсенділігін көрсетті. Микробқақарсы және биоқабықшаның түзілуіне қарсы белсенділіктің кең спектрін ескере отырып, розмарин қышқылын басым қосылыс ретінде және құрамында розмарин қышқылы бар бірқатар дәрілік өсімдіктермен бірге микробқақарсы агент ретінде пайдалануға болады. Розмарин қышқылы стоматологиялық тәжірибеде қолданылатын микробқақарсы және емдік-профилактикалық препараттарды әзірлеу үшін негіз бола алады.

Кілт сөздер: розмарин қышқылы, колориметриялық әдіс, профилометрия, биоқабықшалар, *Streptococcus mutans*.

Я.К. Левая, К.Ж. Бадекова, Г.А. Атажанова

Розмариновая кислота, ингибирующая образование биопленки *Streptococcus mutans*

Возникающая устойчивость к противомикробным препаратам и побочные эффекты, связанные с чрезмерным использованием антибиотиков, обусловливают необходимость в новых противомикробных терапевтических средствах с природными соединениями, рассматриваемыми в качестве привлекательной альтернативы. Розмариновая кислота в изобилии присутствует в лекарственных растениях. Целью данного исследования было выяснить ее возможную роль в качестве ингибитора роста биопленки *S.mutans*. Количество биопленки, образованной бактериями *S. mutans*, оценивали с помощью колориметрического метода и оптической профилометрии. В данном исследовании розмариновая кислота продемонстрировала значительную ингибирующую активность в концентрации 5 мг/мл на образование биопленки *S. mutans* в среде, содержащей 1 % сахарозы. Учитывая широкий антимикробный и антибиопленочный спектры активности, розмариновая кислота может быть использована как антимикробный агент вместе с рядом лекарственных растений, содержащих розмариновую кислоту, в качестве доминирующего соединения. Тем не менее розмариновая кислота может служить основой для разработки антимикробных и лечебно-профилактических препаратов, использующихся в стоматологической практике.

Ключевые слова: розмариновая кислота, колориметрический метод, профилометрия, биопленки, *Streptococcus mutans.*

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