Original Article

Eradication of Biofilm Formation by *Crocus sativus* Alcoholic Extract in *Streptococcus mutans* Clinical Isolates

Running title: Anti-biofilm of *Crocus sativus*

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Abstract

Despite major advances in oral health in the past decades, but tooth decay is one of the most common preventable diseases worldwide [1]. Nowadays, *Streptococcus mutans* is discussed as one of the most important challenges in tooth decay. In the other hand, medicinal herbs can be considered as an effective weapon against infectious diseases. The purpose of the current study was to investigate the biofilm formation in *S. mutans* clinical isolates and to evaluate the anti-biofilm properties of *C. sativus* against *S. mutans* clinical isolates. In this study, thirty dental plaque samples were collected from patients with referring to treatment centers of Hamedan University of Medical Sciences. Then, identification of samples was performed by standard methods. Biofilm formation was evaluated on *S. mutans* clinical isolates. Afterwards, *C. sativus* alcoholic extract was applied as an anti-biofilm formation in *S. mutans* clinical isolates. Our results demonstrated that a significant number of *S. mutans* samples were identified as strong, moderate and week biofilm producers. Then, *C. sativus* in 60 µg/ml, 30 µg/ml and 8 µg/ml were able to eradicated strong, moderate and week biofilm formation in *C. sativus*, respectively. Therefore, the present study offered *C. sativus* as an anti-biofilm formation in *S. mutans* clinical isolates. In addition, more extensive studies and in vivo research are needed to confirm the results of this study.

Keywords: Tooth Decay; *Streptococcus mutans*; Biofilm formation; *Crocus sativus*

Introduction

Despite major advances in oral health in the past decades, but tooth decay is one of the most common preventable diseases worldwide [1]. Tooth decay is very common in developing countries. There are many factors involved in tooth decay such as nutrition, poor oral hygiene and the accumulation of bacteria causing dental plaques [2]. Also, among the microorganisms in the oral cavity, lactic acid producing bacteria such as *Lactobacillus, Bifidobacter* and

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virulent *Streptococcus* are of particular importance. So, *viridans streptococcus* group is more prone to oral flora and tooth decay. One of the important members of virulent *Streptococcus* is *Streptococcus mutans* [3].

*S. mutans* was isolated from dental caries in 1924. Also, it was recognized as a major cause of tooth decay in the mid-1960s [4]. *S. mutans* is capable of producing large amounts of organic acids (acidogenicity). Also, it has the ability to survive in acidic pH and the ability to synthesize external glucan from sucrose [5].

This ability in *S. mutans* plays an important role in the establishment and accumulation of biofilm on the tooth surface. *S. mutans* biofilm plays key role in dental plaque formation and caries [6]. In addition, most infections caused by biofilm formation in bacteria are resistant to treatment. According to previous reports, bacterial resistance in biofilm formation state is 1000 times higher than planktonic state [7]. *S. mutans* not only play a role in dental caries, but also in other diseases such as lip fissure, parotid gland inflammation and etc. In addition, Antimicrobial agents, antibiotics, and antimicrobial mouthwashes on the market have some side effects besides being useful [8].

Therefore, it is necessary to use a herbal medicine that has antimicrobial effects and has minimum side effects [9]. Nowadays, the use of antibiotics as a common treatment against the biofilm structure of bacteria is one of the most important challenges in the field of treatment. New drugs discovery can be a good choice for eradication of biofilm formation [10]. Also, many studies have shown that different types of medical plants can be considered as an effective weapon against infectious diseases. Nowadays, medical plants as a safe way to inhibit the biofilm formation of pathogenic bacteria are essential. Long and light history of using medicinal plants in traditional medicine, the low cost of producing these plants, lack of environmental problems, and absence of drug resistance has made medicinal plants a suitable select for biofilm inhibition in *S. mutans* and other bacteria with capacity produce biofilm formation [11].

In addition, various reports mentioned *Crocus sativus* is a native plant in Iran, India, Greece and Spain. So, from ancient times *C. sativus* was used as food and medicine. In addition, the essential oil of this plant has antibacterial effects [12]. According to previous study, these plants have anti-biofilm properties. So; *C. sativus* contains antimicrobial agents such as *crocin*, *crocetin*, *picrocrocin* and safranal on the biofilm structure of the bacteria [13].

Due to these reasons, in this study, at the first dental plaque samples were collected from patients with referring to treatment centers. Then, biofilm formation in *S. mutans* isolates was investigated. In addition, anti-biofilm properties of *C. sativus* were evaluated on *S. mutans* clinical isolates.

**Method and Materials**

**Bacterial collection and identification**

A total of thirty samples of dental swabs were prepared from the teeth surface of the patients (with patient satisfaction). In this study, patients were referred to the medical centers of Hamedan University of Medical Sciences. Also, patients with malignant and weak immune system and diabetic patients were excluded. In addition, Swab samples were obtained in 2019.

Then, samples were cultured in the medium of blood agar, Metis salivarius agar and Bile esculin Agar. Then, incubation was performed at 37 °C with 5% carbon dioxide. Then, gram staining was performed. Also, Biochemical tests including catalase, oxidase and fermentation of mannose sugars, sorbitol, salicin, trehalose, lactose and mannitol...
were performed. In this study, mannitol, lactose, salicin, and trehalose tests of *S. mutans* were positive, but mannose, catalase and oxidase tests were negative.

Preparation of *C. sativus* extract

Ethanol at 95% concentration was used as the solvent. Ethanolic extracts of *C. sativus* were placed on rotor for 24 hours and was filtered through a filter paper. The filtered extract was subjected to a rotary apparatus, the exit solvent was evaporated and final extract was dried at room temperature.

Cell culture and Toxicity assay

Hence, the cells were inoculated in 96-well micro plates. Cellular density was determined. Then the *C. sativus* ethanol extracts were applied to determine their cytotoxicity effect on a Vero cell line. Then, MTT assay was performed and the absorbance of the transformed dye was measured at a 600 nm wavelength. MTT assay was done by MTT assay kit (Sigma, United States).

Biofilm formation assay

Briefly, 0.5 McFarland solutions of *S. mutans* was prepared using Muller Hinton-broth medium. Then, 200 µL of suspensions of *S. mutans* clinical isolate with broth media (BHI broth supplemented with Sucrose 2%) were inoculated in 96 well polystyrene plates and incubated at 37°C for 48 hours and evaluation of biofilm formation. Broth media were used for negative control and also PTCC 35688 strain of *Streptococcus mutans* positive biofilm was used as a positive control. Then, surplus stain was washed by 200 µl sterile distilled water for three times. After washing wells with distilled water, the entire well was added 200 µl of 95% of ethanol. Hence, all of wells were stained with 200 µl of crystal violet for 20 minutes. Then, surplus stain was washed by 200 µl sterile distilled water for three times. After washing wells with distilled water, the entire well was added 200 µl of 95% of ethanol. Then optical density was measured immediately. Also, Optical densitometry was measured at 550 nm by immunoassay reader. Finally, isolates were divided into three categories according to biofilm formation. These groups including biofilms with 75 percentage of the biomass of the positive control, moderately adherent biofilms with 25-75 percentage biomass or weak biofilms with 25 percentage of the biomass of the positive control.

Semi-quantification of biofilm biomass

In this study, we used the methodology defined by Mowat et al [14].

Anti-biofilm properties determination of *C. sativus*

The bacterial suspension was inoculated in 96 microplates. Different concentrations of *C. sativus* (1-100 µg/ml) were performed. Finally, biofilm formation assay was applied.

Statistical analysis

In this study, statistical analysis was performed by using SPSS (version 24). Also, significant differences were evaluated with using analysis software between the control group and the groups exposed to different concentrations of the *C. sativus* extract.

So, IC50 of *C. sativus* extract against Vero cell line was obtained by prism 6.

In addition, all experiments were performed in triplicate.
Result

Dental swabs sampling

The results of this study showed, the number of *S. mutans* identified in this research was 66.63 % in males and 33.37 % in females. The mean patient age was mentioned in Table A.

<table>
<thead>
<tr>
<th>Frequency percentage</th>
<th>age average</th>
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<tbody>
<tr>
<td>16.66</td>
<td>7-10</td>
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<tr>
<td>16.66</td>
<td>11-20</td>
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<tr>
<td>23.33</td>
<td>21-30</td>
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<tr>
<td>26.66</td>
<td>31-40</td>
</tr>
<tr>
<td>16.69</td>
<td>41-50</td>
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</table>

Table A Results the mean age of patients

Biofilm formation in *S. mutans*

Initially, the bacteria were confirmed by phenotypic methods. Furthermore, we discovered biofilm formations as a significant factor in *S. mutans* clinical isolates; then, we discovered several clinical isolates with a strong biofilm structure (n=8). In high number of *S. mutans* clinical isolates, moderate biofilm formation was also significant (n=12). Nevertheless, there was also *S. mutans* isolate with a weak biofilm formation (n=6). In addition, strains with no biofilm were very low and negligible (n=4). These results were summarized in Fig 1.

![biofilm formation](image)

**Fig. 1** The biofilm formation in *Streptococcus mutans* Clinical isolates.

Cytotoxicity assays of *C. sativus* extracts on a Vero cell line

Cytotoxicity assays were performed on ethanolic seed extract of *C. sativus* and the result was demonstrated IC50 of *C. sativus* is 100 μg / ml.

The effect of *C. sativus* extract on biofilm formation in *S. mutans*
IC$_{50}$ of $C$. sativus was 100 $\mu$g/ml. Different concentration (1-100 $\mu$g/ml) of $C$. sativus was performed for strain with ability of biofilm formation. Finally, $C$. sativus in 60 $\mu$g/ml, 30 $\mu$g/ml and 8 $\mu$g/ml were able to eradicate strong, moderate and week biofilm formation in $C$. sativus, respectively (Fig2).

![Anti-biofilm of Crocus sativus](image)

**Fig. 2** Anti-biofilm properties of $C$. sativus in $S$. mutans clinical isolates.

**Discussion and Conclusion**

Nowadays, Dental caries is a microbial disease which is one of the most prevalent oral infectious diseases in developing countries. Dental caries is a multifactorial infectious disease. The primary cause of dental caries is dental plaque which is a complex biofilm [15]. Biofilm formation in bacteria can create serious challenges in the fight against infectious diseases. Also, the biofilm formation can harmful to human health [16]. In fact, one of the main mechanisms of survival of bacteria in different environments is the ability to biofilms formation [17]. In addition, bacteria capable of producing biofilms can escape the host immune system and thus cause chronic infections [18]. One of the contributing factors to chronic infections is the formation of biofilm structure in bacteria [19]. $S$. mutans is the most important etiologic agents in the development of biofilm formation for survival and persistence in dental plaque [20]. Medicinal plants extracts have a long history of use for treat various diseases [21]. Though, valid scientific research confirmed the properties of $C$. sativus. In addition, the essential oil of this plant has antibacterial effects [22]. In this study, our data demonstrated that biofilm formations as a significant factor in $S$. mutans clinical isolates. Furthermore, our results declared that medicinal plants can be used as a suitable candidate for the treatment of biofilm formation caused by $S$. mutans. However, it seems that studies in vivo and broader studies in this context were necessary.

**Conflict of Interest**

The author has no conflicts of interest.
References
