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Inhibition and Eradication of Oral *Streptococcus gordonii* Denture Biofilms Using Extracts of *Salvadora persica* and *Juglans regia*

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Abstract

Oral biofilms formed by *Streptococcus gordonii* on removable dentures pose significant challenges, particularly for elderly individuals. Natural plant extracts, such as those from *Salvadora persica* and *Juglans regia*, have been explored for their potential antibacterial and antibiofilm activities. The biofilm-forming ability of *S. gordonii* isolated from elderly dentures was qualitatively and quantitatively assessed. The antibacterial and antibiofilm activities of *S. persica* and *J. regia* extracts were evaluated using a broth microdilution assay. Additionally, the effect of the extracts on artificial biofilms adhered to resin composite surfaces was examined and visualized using an environmental scanning electron microscope. Data revealed that *S. gordonii* biofilms adhered to artificial surfaces were less sensitive to the plant extracts compared to their planktonic counterparts. However, both planktonic and biofilm forms of *S. gordonii* exhibited increased susceptibility in the presence of *J. regia* extract, which demonstrated higher activity than *S. persica*. These findings highlight the potential of *J. regia* and *S. persica* extracts as natural antiplaque agents, particularly in oral care products designed for elderly denture wearers. Further research is warranted to explore their applications in daily oral hygiene.

Keywords: Biofilm, Dentures, Antibacterials, *Streptococcus*, *Juglans regia*, *Salvadora persica*.

Introduction

Wearing removable dentures induces local environmental changes, leading to an imbalance in the local microbiota.[1] It has been extensively documented that denture biofilm acts as a reservoir for opportunistic microorganisms that can cause local infections, especially in the elderly who do not keep their dentures sufficiently clean.[2] There is a lack of consensus around suitable cleaning agents, with many denture wearers opting to use toothpaste to mechanically clean their dentures. However, this has been shown to induce abrasions, resulting in physical defects on the denture acrylic that may lead to enhanced microbial adhesion through altered surface topography.[3] Moreover, pathogenic microorganisms have increasingly developed resistance against conventional chemical agents, which highlights the importance of exploring plant extracts as alternative antimicrobial agents.[4]

Chewing sticks may play a role in the promotion of oral hygiene. Further evaluation of their effectiveness is essential, as stated in the Consensus Report on Oral Hygiene.[5] The traditional medicinal use of *S. persica* as an antimicrobial stick toothbrush for oral hygiene and to treat gum inflammation is a centuries old practice and a part of the Greco-Arab system of medicine. *S.persica* is a member of the Salvadoraceae family, the properties of this stick are mainly due to the Benzyl Isothiocyanate it contains.[6] In addition, inorganic elements such as fluoride, calcium, and phosphorus, along with other bioactive metabolites like alkaloids, acids, tannins, saponins, and vitamin C, contribute to the effectiveness of this oral tool.[7] *Juglans regia* or walnut species, belong to the Juglandaceae family, is a medicinal plant that has been used in traditional medicine for the treatment of a lot of diseases.[8] Preliminary screenings carried out on the bark of *J.regia* revealed the presence of several phytochemical constituents, including high concentrations of alkaloids, coumarins, quinones, terpenoids, saponins, amino acids, and proteins, with lower concentrations of tannins and anthraquinones. Interestingly, flavonoids were absent from the bark, unlike in the leaves. Therefore, this study aims to investigate the

antibacterial activity of extracts from two Algerian chewing sticks on the growth of oral bacteria, both in their planktonic forms and within denture biofilms.

Materials and Methods

Sampling and identification

A sterile cytobrush was used to collect samples from the intrados of removable dentures of patients undergoing treatment at Tlemcen University Dental Clinic, Algeria. Specimens were inoculated onto Columbia ANC agar (Liofilchem, Italy) supplemented with 5% (v/v) blood. The plates were incubated at 37°C for 48h under aerobic conditions. For identification, isolates were initially identified on the basis of colony morphology, negative catalase reaction, Gram's stain, API STREP (bioMérieux, France), and Matrix Assisted Laser Desorption Ionization Time of Flight Mass Spectrometry (MALDI-TOF MS) automatic identification.

Biofilm formation

Two phenotypic methods were used to detect biofilm production. The extracellular polysaccharide substance and slime-producing ability of the isolates were determined with the Congo Red Agar (CRA) method,[9] and the modified tissue culture plate (TCP) test was used for the quantification of biofilm.[10]

Extracts preparation

J. regia roots and *S. persica* branches as the plant materials were kept dried for 2 weeks at 30°C before extract preparation. Extract was prepared according to Darmani *et al.*[11] In brief, ground chewing sticks samples were extracted in 80% methanol. The mixture was left for 3 days at room temperature and then filtered using Whatman N^{o} 4 filter paper (Whatman, England). The ethanolic extract was then concentrated and stored at 4°C.

Antibacterial activity

The antibacterial tests of the extracts studied were carried out using the agar medium diffusion technique, which is a qualitative technique based on measuring the diameters of the inhibition zones according to Gulluce *et al.* [12]

Planktonic growth and biofilm inhibition ability

Minimum inhibitory concentrations (MICs) were determined by the microtiter broth method, in sterile flat bottom 96-well plates.[13] Serial dilution techniques were used to determine the MIC_{50} and MIC_{90} of extracts at concentrations of 51.2mgmL^{-1}. The mean % inhibition of replicate tests was used to determine the final MIC values.

$$\% \text{ d'inhibition} = [\, 1 - ((OD_{t24} - OD_{t0}) / (OD_{c24} - OD_{c0}))\,] \times 100$$

OD_{t24} = optical density of the test well at 24h of incubation
OD_{t0} = optical density of the test well before incubation
OD_{c24} = optical density of control well at 24h of incubation
OD_{c0} = optical density of control well before incubation

Optical density of each well was measured at 490nm using a microtiter reader (ELx808IU, BioTek Instruments Inc., Winooski VT, USA).

Antibiofilm activity

A modified microtiter plate test [14] was used to test the effect of plant extracts on biofilm formation. Negative controls (cells +BHIB), vehicle controls (cells + BHIB + DMSO), and media controls (BHIB) were included. Biofilms were established in the 96-well plates for 24h at 37°C. At 24h after incubation, the appropriate concentration of test extract was added. The adherent biofilm was stained with 150 mL of crystal violet (1%) for 15min at room temperature. Excess stain was rinsed off and the dye was re-solubilized with 150 mL of 95% ethanol per well. The concentration at which the extract depleted the biofilm biomass by at least 50% was labeled as the IC_{50}.

$$\% \text{ Inhibition} = [(OD_t - OD_c) / OD_v] \times 100$$

OD_t = optical density of the test well at 24 h of incubation.
OD_c = optical density of control well at 24h of incubation.

OD_v = optical density of the vehicle control at 24h of incubation.

Resin attachment

To evaluate the effects of *J. regia* and *S. persica* extracts on bacterial biofilm on resins, uniform-sized acrylic resin plates (10×10×1) mm^3 were prepared, sterilized, and immersed in 5mL of a 0.5Mc Farland bacterial suspension. After incubation for 48h at 37°C, each plate was rinsed twice in sterile saline, then placed in contact with each of the two extracts at a concentration equal to the MIC already determined. After eight hours of contact (estimated as overnight soaking time), each plate was sonicated in 10mL of sterile saline, of which a volume of 100µL was spread on Müller-Hinton agar. After incubation for 48h at 37°C, bacterial cell counts were performed. All results were expressed as percentage of bacterial survivor, and compared to the control incubated with saline solution.[15]

Electronic microscopy

Electronic microscopy was carried out to observe the effect of extract on *S. gordonii* adhesion to resin, after 4h of treatment, using an environmental scanning electron microscope (XL 30 ESEM Philips, Eindhoven, Netherlands). Sterile acrylic resin plate were immersed in a bacterial suspension and incubated for 24 hours at 37°C.

Results and Discussion

Removable dental prosthesis, such as full or partial dentures, or orthopedic appliance will create new surfaces for biofilm formation and this increases the total amount of biofilm dramatically.[16] Patients should be educated on good denture hygiene and cleaning practices because poor hygiene is a major factor disposing to development of diseases as it allows biofilm formation.[2]

Bacterial identification and biofilm formation ability

Bacterial identification observed by isolations were found to be gram-positive rods in chains and catalase negative, which were presumed to be *Streptococcus* spp. The identity of the selected isolate *S. gordonii* was confirmed by the analytical profile index (API) system and

MALDI-TOF results. CRA plate method and confirmation by microtiter plate assay revealed the biofilm-forming abilities (Table 1). *S.gordonii* is part of Viridans streptococci that comprise a vital part of the normal flora of the human upper respiratory tract,[17] and belong to the Mitis group, pioneer species in the mouth of newborns (primary colonizer) that allow the assembly of a complex microbiota that predominate the early formation of dental plaques.[18] It is also known as an opportunistic pathogen that easily attaches to host tissues, including dental surfaces and heart valves, forming biofilms that can cause local or systemic diseases, such as apical periodontitis and infective endocarditis. [19]

Table 1. Bacterial identification results and biofilm formation ability

Bacteria strain	Hemolysis	Biofilm (TCP)	Slime (CRA)	Denture (Sample)
Streptococcus gordonii	Gamma	Strong producer	Variable	Used for 24years Brushing with tap water once a week Night wear

An ideal denture cleanser should be able to reduce biofilm without affecting the physical and mechanical properties of the denture base or denture teeth (No bleaching or abrasion). It must be non-toxic, short-acting, easy to use and cost-effective.[2,20] Investigations on antibacterial plants have focused entirely on planktonic bacteria with little emphasis on the more resistant and difficult to control biofilm forms.[21] For these reasons and with the purpose of evaluating another potential alternative substance, with a possible application in dentures desinfection, two plant species of the Algerian traditional medicine were selected Swek (*Juglans regia*) and Miswek (*Salvadora persica*) , based on their popular use, for antibacterial activity.

Antibacterial activity

The diameters of the inhibition zones of the oral bacteria tested in the presence of the extracts are recorded in Table 2. It appears from the results that the two extracts have an inhibitory effect on the bacterial strains. *J. regia* extract shows good inhibitory activity compared to *S. persica* extract. The antimicrobial activity observed during the present study is in agreement with the results of previous investigations where *S. persica* exhibited significant antimicrobial activity against streptococci. Chelli-Chentouf *et al.*[22] showed that *S. persica* from Algerian Hoggar had an inhibitory effect on the growth of *S. mutans* with an inhibition diameter of 7.5mm for a concentration of 400mgmL^{-1} in methanolic extract. The work of Balto *et al.*[23] found that the ethanolic extract exhibited an increased inhibitory effect against *Streptococcus* species where the extract at 100mgmL^{-1} was associated with a large inhibition zone of 20 mm against *S. sanguis* and thus comparable to chlorexidine 0.2%. More recently, the results of *in vivo* and *ex vivo* experiments of Abdeltawab *et al.*[24] indicated that the concentration of 100% methanolic extract of *S. persica* has effective antimicrobial activity and the largest zone of inhibition was observed after 3 days (15 mm in diameter) for *S. mutans*.

Table 2. Inhibition zones of *S. persica* and *J. regia* alcoholic extracts

Bacterial strain	Inhibition zones diameters (mm)	
	S. persica	*J. regia*
Streptococcus gordonii	13 ± 1	15.3 ± 1.5

Overall, the data from this experiment clearly indicated that *Juglans regia* and *Salvadora persica* extracts inhibited the growth of the bacteria tested. However, the difference in activity between the extracts of the plants studied is probably dependent on the nature of the molecules contained in each of them or on the ability of these molecules to diffuse uniformly in the agar. Indeed, the optimal effectiveness of an extract may not be due to a majority active constituent, but rather to the combined action (synergy) of different compounds.[25]

Biofilm and planktonic growth inhibition

Data presented in Figure 1 revealed MIC values showing that the activity of *J. regia* extract was more pronounced against *S. gordonii* planktonic growth with an $IC_{50} \geq 0.8$ $mgmL^{-1}$, than *S. persica* extract ($IC_{50} \geq 6.4$ $mgmL^{-1}$). The MIC value of *S. persica* extract were lower than those obtained in a recent study carried out by Chelli-Chentouf *et al.*[22] they showed that the MIC values of methanolic extract varied with the test microbe from 100 to 400$mgmL^{-1}$. However, the study of Al-Sohaibani and Murugan [26] showed a lower MIC (2.6 $mgmL^{-1}$) for the Saudian *S. persica*. In a recent study, the essential oils of dried and fresh roots of *S. persica* showed IC_{50} values comparable to those of chlorhexidine digluconate.[27] Furthermore, a comparative study found that the aqueous extracts of miswak and derum (different type of chewing stick obtained from walnut tree *J. regia*) were both able to significantly inhibit the growth of cariogenic bacteria,[28] with the derum extract showing greater activity than miswak, which is comparable to our study results. Another study conducted by Sharafati-Chaleshtori *et al.*[29] where the ethanolic extract of the walnut leaves had shown a significant potential and an MIC of 15.6 $mgmL^{-1}$ for *S. salivarius* and *S. sanguinis*. Many studies already conducted on different extracts of bark leaves, fruits and even flowers of *J .regia* from different countries, have revealed a broad spectrum antibacterial activity against gram-positive and gram-negative bacteria thus supporting its application as a preventive remedy against various microbial diseases of the oral cavity.[30-33] The study of Jahanban-Esfahlan *et al.*[34] estimates that this antimicrobial activity is mainly due to the chemical composition of *J. regia* in essential oils, the main components of which are phenolic compounds, terpenoids, alkaloids, flavonoids and steroids.

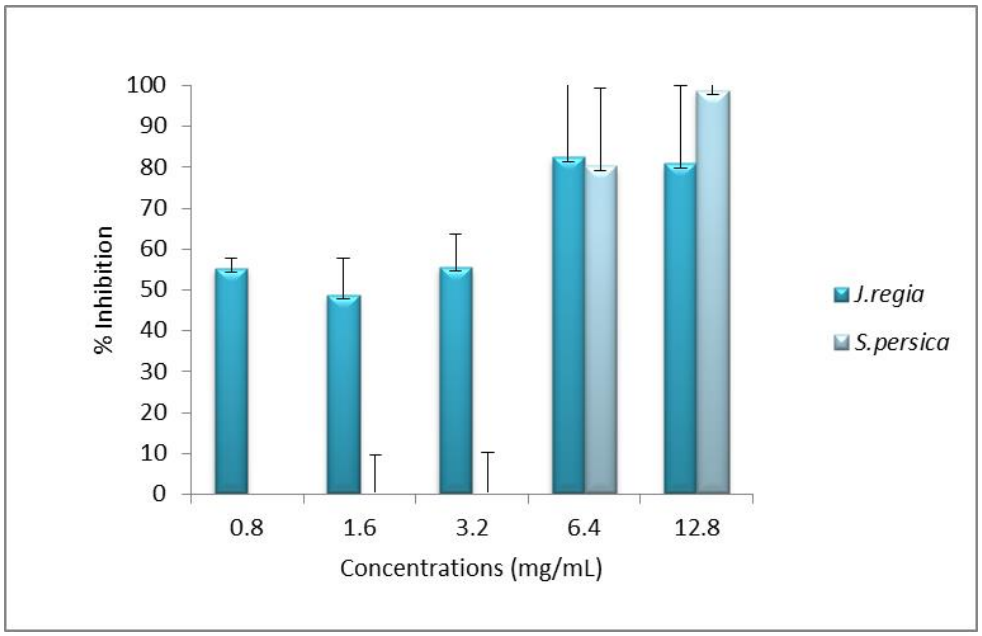


Figure 1: The percent inhibition of *Juglans regia* and *Salvadora persica* extracts for planktonic growth of *Streptococcus gordonii* strains. Data expressed as mean ± standard deviation (SD). Error bars represent SD.

Antibiofilm activity

Aiming at restoring the natural antimicrobial capacity of removing adhered bacteria, *in vitro*, two methods were used to evaluate the antibiofilm activity, the crystal violet assay and the adhesion assay on resin. Both methods showed a good antibiofilm activity exhibited by the two plants. These observations are speculated by the fact that extracts efficiently penetrate in the matrix of extracellular polymeric substances and kill or remove the biofilm-embedded cells. The effect of methanolic extract on the attachment and biofilm formation on microtiter plates was investigated. The *J. regia* and *S. persica* extracts showed an $IC_{50} \geq 51.2$ $mgmL^{-1}$ for biofilm inhibition. None of the two extracts were able to eradicate biofilm cells completely (Figure 2). Extracts were effective in inhibiting biofilm formation and planktonic bacterial growth at low concentrations, but biofilm bacteria appeared 8 to 60 times more resistant to *S. persica* and *J. regia* extracts, respectively. Crystal violet staining showed that the two extracts exhibit antibiofilm activity by 50%, whereas the previous investigation conducted by Al-Sohaibani and

Murugan[26] revealed a significant reduction of sessile cells, and biofilm formation was inhibited up to 87.92%, and it also indicated that *S. persica* contains bioactive antibiofilm agents with dual functionalities of growth inhibition and quorum sensing regulator interaction. According to Balhaddad *et al.*[35] the concentration of 10 mgmL^{-1} of the methanolic extract of *S. persica* was determined as the MIC of biofilm. This difference in the antimicrobial activities may be attributed to differences in the timing and location of plant collection, extraction methods, variability in the tested bacterial species, and the evaluation techniques. Many researchers have studied the constituents of miswak and found it to contain more than 10 different natural chemical compounds, all considered essential for good oral and dental hygiene.[7,36,37] The study of Abdulbaqi *et al.*[38] demonstrated that the mixture of *S. persica* root stick and green tea (*Camellia sinensis L*. leaves) exhibited synergistic effects against primary colonizers of dental plaque and antiplaque activity *in vitro*. This can potentially produce novel effective poly-herbal formulations for oral care.

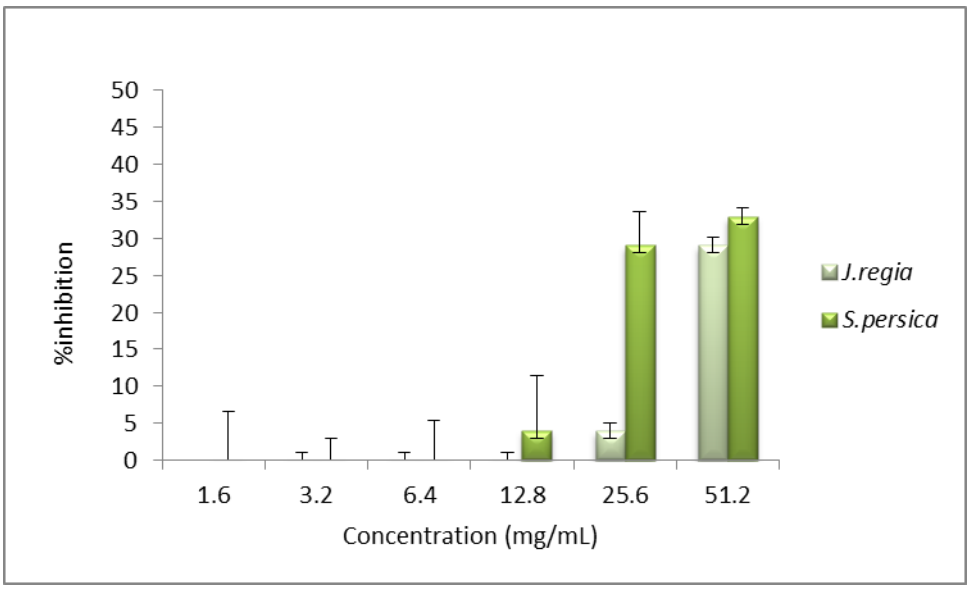


Figure 2: The percent inhibition of *Juglans regia* and *Salvadora persica* extracts for *Streptococcus gordonii* biofilm. Data expressed as mean ± standard deviation (SD). Error bars represent SD.

Bacterial attachment on acrylic resin was observed after 8h in contact with the extracts, a reduction in biofilm microbial counting was observed for the incubated systems (Figure 3). *S. persica* extract was found to be effective against biofilms, reducing the microbial count by approximately 99%. Whereas *S. gordonii* showed a complete susceptibility to *J. regia* (100%) (Figure 3). Treated resin showed the number of viable cells statistically smaller than that of control. This showed that the number of bacteria covered by biofilm was smaller over time with the fact of an atypical evolution of biofilm formation stages.

The battle against oral biofilms is a very challenging task, mainly due to their tendency to persist in spite of treatment bacterial cells in the biofilm exhibit low metabolic activity, strong drug tolerance, and specific phenotype changes caused by the cell signaling or cross-species reciprocal protection.[39] Consequently, biofilm microorganisms can be up to 1000 times more resistant than planktonic bacteria to conventional antimicrobial therapies with antibacterial agents such as antibiotics or chlorhexidine. [40,41]

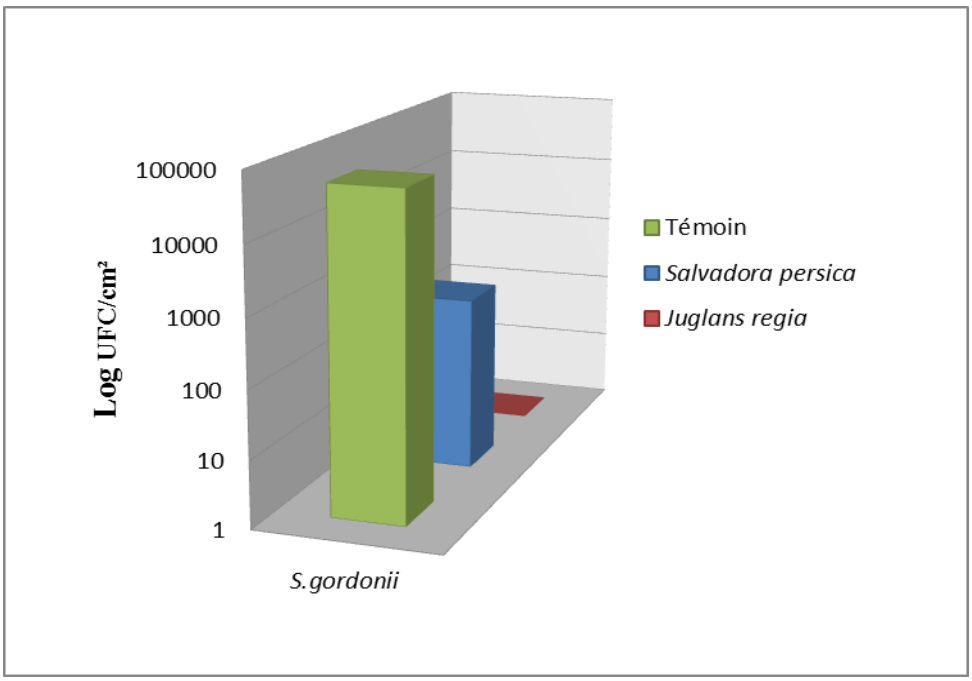
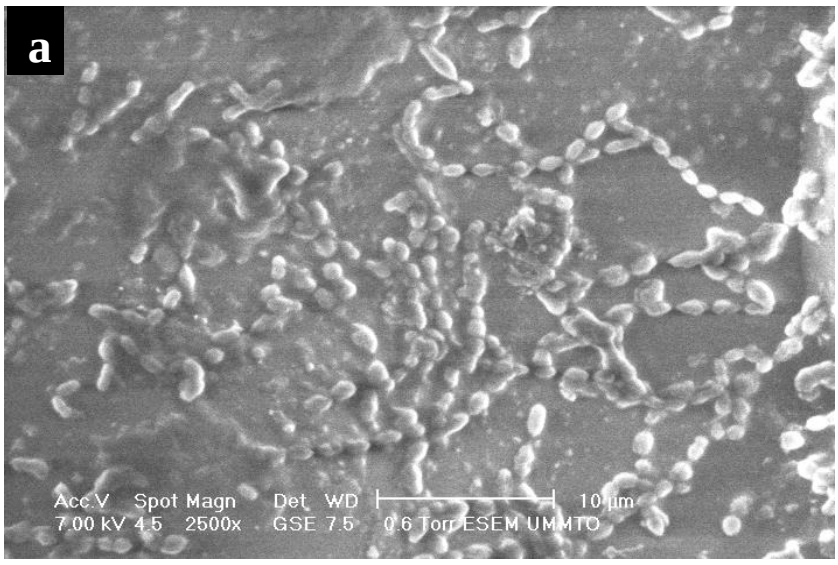


Figure 3: Activity of *Juglans regia* and *Salvadora persica* extracts on Streptococcal resin biofilm.

Microscopy

In agreement with viable counting cells, ESEM image showed clear effects of these agents on *S. gordonii* growth in terms of decreased attachment (Figure 4). Microscopic observations gave further evidence that the extract may not only inhibit the attachment and aggregation of the cells but also act on the bacterial membranes, after only 4 hours of treatment with both extracts. Microscopic examination revealed the presence of elongated cells with interrupted chains after treatment with *S. persica* extract (Figure 4b). Images of *S. gordonii* cells incubated with *J. regia* extract revealed numerous structural changes in cell morphology. These were damaged and shredded with distorted contours which led to subsequent leakage of cellular contents (Figure 4c). At the same time, it is worth mentioning that, while in the treated biofilm several gaps were observed, the untreated control cells showed the typical streptococcal appearance in the form of cocci the biofilm appeared as a compact structure with bacteria close to each other (Figure 4a).



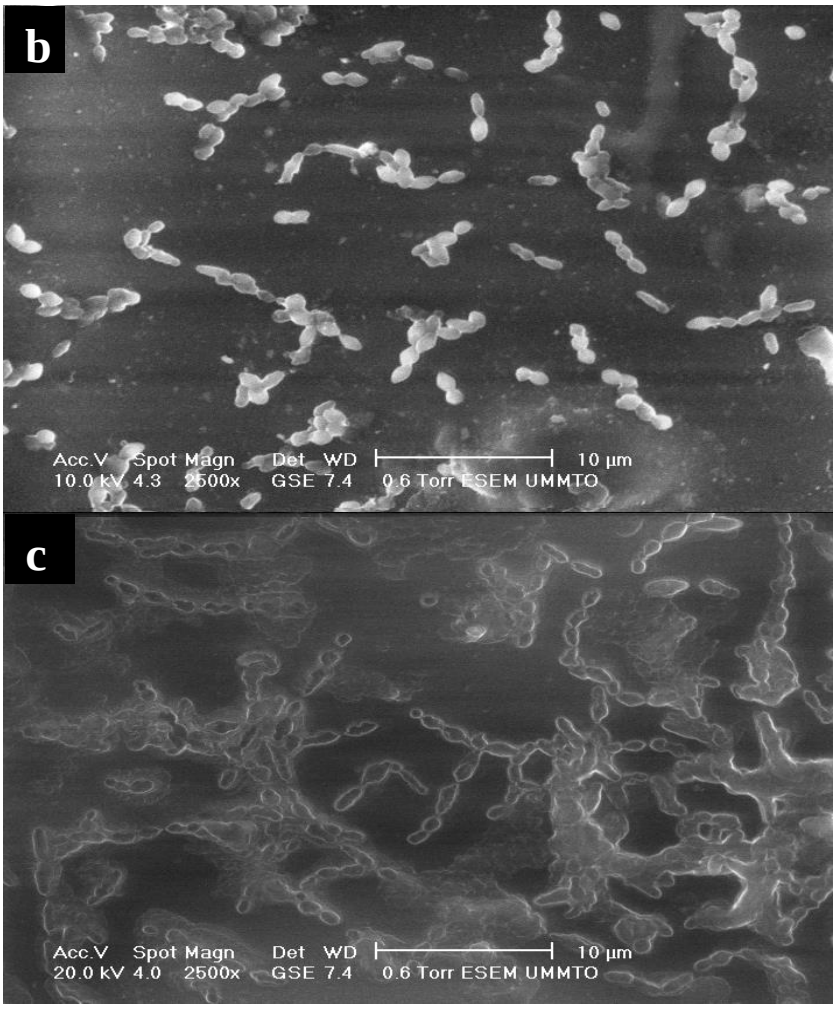


Figure 4: Environmental scanning electron microscope (ESEM) images showing the effect of extracts on *Streptococcus gordonii* biofilm formed on acrylic resin (scale bars = 10 µm). (a) *S. gordonii* biofilm formed on resin before antimicrobial treatment. (b) *Salvadora persica* extract effect. (c) *Juglans regia* extract effect

Although this study provided important insights, certain limitations should be acknowledged. First, the experiments were conducted under in vitro conditions, which may not fully replicate the complexity of the oral environment, including saliva dynamics, diet, and interactions with other microbial species. Second, the variability in bioactive compound concentrations due to differences in plant harvesting conditions, such as geography and seasonality, was not considered and may affect reproducibility. Third, the study focused exclusively on *Streptococcus gordonii* as a model organism; further investigations should include a broader

range of oral pathogens and polymicrobial biofilms typically associated with denture plaque. Finally, potential toxicity, biocompatibility, and the long-term effects of these extracts on oral tissues and prosthetic materials remain unexplored, necessitating further research to ensure their safe application.

Conclusion

The present study highlights the antibacterial and antibiofilm properties of *Salvadora persica* and *Juglans regia* extracts against *Streptococcus gordonii*, a key oral biofilm-forming microorganism. Both extracts demonstrated significant inhibitory effects on planktonic bacterial growth and biofilm formation, supporting their potential use in denture hygiene management. These findings underscore the promise of plant-based natural products as effective, alternative strategies for denture cleaning and oral health maintenance.

In terms of future perspectives, this work opens avenues for isolating and characterizing the bioactive compounds responsible for these effects to better understand their mechanisms of action. Moreover, the integration of such extracts into novel oral care products—such as denture cleansers, oral rinses, or antimicrobial coatings for prosthetic devices—could provide accessible, eco-friendly alternatives to conventional chemical agents. Conducting in vivo studies and evaluating their efficacy in diverse oral environments and microbiomes will be crucial steps toward clinical translation.

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None.

Conflicts of interest

The authors declare no conflicts of interest.

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