ORIGINAL ARTICLE
ISSN 2645-4351
https://doi.org/10.22037/jds.v43i2.46679

Antibacterial Effects of Ascophyllum Nodosum and Punica Granatum Extracts on Streptococcus Mutans and Lactobacillus Acidophilus

Mahsa Ladanmoghaddam[a], Bafrin Fattahi[b], Parisa Aref[c], Sara Tavasoli Hojjati[c], Fatemeh Emadi[d], Elahe Motevaseli[e]

[a]Post graduated dental student, Department of Oral Medicine, School of Dentistry, Shahid Beheshti University of Medical Sciences, Tehran, Iran.
[b]Post graduated dental student, Department of Endodontic, School of Dentistry, Tabriz University of Medical Sciences, Tabriz, Iran.
[c]Assistant Professor, Pediatric Dentistry Dept., Faculty of Dentistry, Tehran Medical Science, Islamic Azad University, Tehran, Iran.
[d]Department of Traditional, Persian medicine, School of medicine, Shahed university,Tehran, Iran.
[e]Department of Molecular Medicine, School of Advanced Technologies in Medicine, Tehran University of Medical Sciences, Tehran, Iran.
*Correspondence to: Parisa Aref, Email: parissa234@yahoo.com

Submitted: 12 November 2024
Revised: 22 January 2025
Accepted: 2 February 2025
Published: Spring 2025

How to cite:
Ladanmoghaddam M, Fattahi B, Aref P, Tavasoli Hojjati S, Emadi F, Motevaseli E. Antibacterial Effects of Ascophyllum Nodosum and Punica Granatum Extracts on Streptococcus Mutans and Lactobacillus Acidophilus. J Dent Sch 2025;43(2):70-77.

Abstract:

Objective(s): This research investigated the influence of Ascophyllum nodosum (A. nodosum) and Punica granatum (P. granatum) extracts on Streptococcus mutans (S. mutans) and Lactobacillus acidophilus (L. acidophilus). **Methods:** In this laboratory-based study, the antibacterial effects of aqueous-acidic, ethanolic, and methanolic extracts of A. nodosum, along with the hydroalcoholic extract of P. granatum were analyzed against S. mutans and L. acidophilus using the disc diffusion method. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the extracts were determined through broth microdilution and macrodilution techniques. As a positive control, 0.2% chlorhexidine (CHX) was utilized, and all experiments were performed in triplicate. Statistical analyses included ANOVA, the Bonferroni test, and Kruskal-Wallis at $p<0.05$. **Results:** CHX exhibited the highest inhibitory and bactericidal effects on both bacterial strains. While both extracts showed antibacterial potential against both organisms, their efficacy was significantly lower than that of CHX ($P<0.05$). The following CHX, aqueous acidic extract of A. nodosum and the hydroalcoholic extract of P. granatum demonstrated superior MIC and bacteriostatic activity against S. mutans. For L. acidophilus, the hydroalcoholic extract of P. granatum showed the best results.
Conclusion: The results indicated that extracts from A. nodosum and P. granatum possess considerable antibacterial properties against S. mutans and L. acidophilus.

Keywords: Punica Granatum; Ascophyllum Nodosum; Streptococcus Mutans; Lactobacillus Acidophilus.

Introduction

Microbial plaque is a major factor in the onset of dental caries, and researchers have been actively seeking natural compounds to address its chemical removal. Routine and accurate mechanical plaque elimination remains the cornerstone for preventing and managing nearly all types of chronic periodontal conditions. Currently, toothbrushes and toothpaste are considered the most effective and practical tools for controlling supragingival plaque.[1]

However, in individuals with sensory or motor impairments, eliminating plaque can be notably challenging, if not impossible. Toothbrushing in difficult-to-access areas also poses significant hurdles. If bacterial plaque is not controlled or removed, it can result in progressive plaque accumulation, ultimately causing tooth decay, bone deterioration, and even tooth loss. Thus, the development of safe and effective adjunctive methods for plaque management is crucial.[2,3] Antimicrobial mouth rinses are widely used to combat plaque buildup.[4,5]

Streptococcus mutans (S. mutans) is a Gram-positive bacterium that plays a central role in initiating caries. Meanwhile, Lactobacillus acidophilus (L. acidophilus), another Gram-positive microorganism, is known for converting sugars into lactic acid[6]. This bacterium is closely linked to the progression of caries and is commonly found in deeper carious lesions. Simark-Mattsson et al. [7] reported isolating L. acidophilus from individuals with extensive dental caries.

Chlorhexidine (CHX) is the most widely used antibacterial mouthwash[8], with its effectiveness in reducing plaque and oral microorganisms well-established in prior studies.[9-12] Nevertheless, CHX use is associated with adverse effects such as tooth discoloration, altered taste, oral mucosal burning, and disruption of the oral microbiome. Consequently, efforts are underway to identify alternative mouthwashes that effectively manage plaque while minimizing side effects.[13]

Research indicates that Ascophyllum nodosum (A. nodosum) can notably reduce supragingival plaque and

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gingival bleeding.[14]However, studies on A. nodosum are limited, primarily focusing on in vivo assessments of A. nodosum tablets[15]To the best of the authors' knowledge, its in vitro antimicrobial properties have not yet been explored.

Meanwhile, Punica granatum (P. granatum), or pomegranate, is recognized for its antimicrobial potential, which is largely attributed to its internal white layer, red outer skin, and seeds. Yet, few studies have investigated the antibacterial efficacy of the entire fruit extract against S. mutans and L. acidophilus.

Given the scarcity of data in this area, the current study sought to evaluate the impact of A. nodosum and P. granatum extracts on S. mutans and L. acidophilus, two key bacterial components of dental biofilm.[16]

Methods

This in vitro experimental study utilized standard bacterial strains of Streptococcus mutans (ATCC35668) and Lactobacillus acidophilus (ATCC4356), which were obtained from the Iranian Culture Collection of Industrial and Infectious Microorganisms. Based on previous research [17-19], the sample size was determined, with each test repeated three times using 96-well plates for different concentrations.

Preparation of A. nodosum extracts: The dried powdered leaves of Ascophyllum nodosum, without additives, were procured from Proden Plaque Off Company. Using the ultrasound-assisted extraction technique described in prior studies [20,21], the aqueous-acidic, ethanolic, and methanolic extracts were prepared. For the aqueous-acidic extract, water and 0.1 M hydrochloric acid were utilized, while 100% ethanol and 100% methanol were used for the ethanolic and methanolic extracts, respectively. The extraction process involved heating the mixtures in an ultrasonic device at 70°C for 15 minutes. [22]The resulting extracts were filtered through Whatman filter paper (Mahstan Chemie, Iran), concentrated, dried, and subsequently diluted to prepare different concentrations.

Preparation of the hydroalcoholic extract of P. granatum: Three medium-sized pomegranates (including their external red peel, inner white layers, and seeds) were finely chopped and blended. A water-ethanol (50:50) solvent was added to the mixture, which was then filtered after 24 hours. The obtained hydroalcoholic extract was heated at 60°C and concentrated to a honey-like consistency. This concentrate was used to create the required dilutions for the study.[22]

Microbial species and culture conditions: Bacterial strains were cultured in brain heart infusion (BHI) broth under anaerobic conditions in a Gas-Pak jar at 37°C for 24 hours. Blood agar was used for streak culturing, with fresh bacteria prepared weekly for testing.

Antibacterial activity evaluation: The antibacterial effects of the extracts were initially assessed using the disc diffusion method. After 24 hours of incubation in blood agar, the bacteria reached peak confluence. Saline was used to prepare bacterial suspensions adjusted to a 0.5 McFarland standard concentration (equivalent to 1.5×10^8 CFU/mL). Bacteria were streak-cultured on blood agar using a sterile swab, and five types of discs were prepared:

1. Positive control disc with 0.2% chlorhexidine (CHX).
2. Test discs containing 100% aqueous-acidic, ethanolic, and methanolic extracts of A. nodosum.
3. Test disc with hydroalcoholic extract (50:50) of P. granatum.
4. Blank disc as a negative control.

All discs were placed on the culture medium and incubated at 37°C for 16 hours (Memert, Germany). After 24 hours, the diameter of the growth inhibition zones was measured and reported in millimeters [23-25]. Changes less than 1 mm were considered negligible. Experiments were conducted in triplicate under aseptic conditions to ensure reliability.

Determination of MIC and MBC: The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the extracts were determined using broth microdilution and macrodilution methods. BHI broth was used, with a bacterial suspension of 0.5 McFarland standard concentration (1.5×10^8 CFU/mL). For the broth microdilution test, 96-well plates were employed, and 100 µL of BHI broth was added to each well. Serial dilutions of the extracts were prepared, and 100 µL of bacterial suspension was introduced into each well. The process was repeated for CHX (positive control).

For the broth macrodilution method, similar steps were followed, with larger volumes in mini-tubes. Positive control tubes (BHI broth with bacteria) and negative control tubes (BHI broth only) were included. The plates and tubes were incubated under anaerobic conditions at 37°C, and turbidity was assessed after 24 hours to determine the MIC. The first clear tube indicated the MIC (mg/mL). To determine the MBC, cultures from tubes immediately before the first clear tube were streaked on blood agar. Plates were incubated at 37°C for 24 hours, with the absence of bacterial growth indicating the MBC (mg/mL). [17, 24-27]

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Statistical analysis: Data were analyzed using SPSS version 20 (SPSS Inc., IL, USA) with ANOVA, the Bonferroni test, and Kruskal-Wallis at a significance level of 0.05.

Results

Table 1 illustrates the diameters of growth inhibition zones for S. mutans and L. acidophilus caused by various extracts. Overall, CHX produced the largest growth inhibition zones. Following CHX, the methanolic extract of A. nodosum and the hydroalcoholic extract of P. granatum exhibited the strongest antibacterial effects against both S. mutans and L. acidophilus, resulting in prominent growth inhibition zones.

Table 1- Mean±SD diameter of the growth inhibition zones for S. mutans and L. acidophilus caused by different extracts

Extract type	Growth inhibition zone for S. mutans (mm)	Growth inhibition zone for L. acidophilus (mm)
Aqueous-acidic extract of A. nodosum	8.833±0.289	10±0. 5
Ethanolic extract of A. nodosum	10.000±0.500	9.833±0.289
Methanolic extract of A. nodosum	13.833±0.289	18.667±0.289
Hydroalcoholic extract of P. granatum	12.000±0.500	17.500±0.5
Chlorhexidine	16.167±0.289	25.667±0.289

The growth inhibition zone diameters for all groups followed a normal distribution, and the assumption of variance homogeneity was satisfied. Consequently, ANOVA was employed for statistical analysis, which identified a significant difference in the growth inhibition zones of S. mutans and L. acidophilus caused by various extracts and CHX (P=0.0001). As a result, pairwise comparisons were performed using the Bonferroni test. Table 2 presents these pairwise comparisons for the growth inhibition zones of S. mutans induced by different extracts and CHX. The analysis revealed significant differences in the inhibition zone diameters among the extracts and CHX (P<0.05). The order of inhibition zone diameters was as follows: aqueous-acidic extract of A. nodosum < ethanolic extract of A. nodosum < hydroalcoholic extract of P. granatum < methanolic extract of A. nodosum < CHX.

Table 3 presents pairwise comparisons of the growth inhibition zones for L. acidophilus produced by four different extracts and CHX. The results revealed significant differences in the inhibition zone diameters between the extracts and CHX (P<0.05), with the exception of the methanolic and ethanolic extracts of A. nodosum (P>0.05). The ranking of growth inhibition zone diameters was as follows: ethanolic extract of A. nodosum < methanolic extract of A. nodosum < aqueous-acidic extract of A. nodosum < hydroalcoholic extract of P. granatum.

Table 2- Pairwise comparisons of the diameter of growth inhibition zones for S. mutans caused by different extracts and CHX using the Bonferroni test

Dependent Variable	Group (I)	Group (J)	Mean Difference (I-J)	Sig.
Diameter of growth inhibition zone	AU	AE	-1.17	0.042
		AM	-5	0.0001
		ME	-3.17	0.0001
		CHX	-7.3	0.0001
	AE	AM	-3.83	0.0001
		ME	-2	0.001
		CHX	-6.17	0.0001
	AM	ME	1.83	0.002
		CHX	-2.33	0.0001
	ME	CHX	-4.17	0.0001

AU: Aqueous-acidic extract of A nodosum; AE: Ethanolic extract of A. nodosum; AM: Methanolic extract of A. nodosum; ME: Hydroalcoholic extract of P. granatum; CHX: Chlorhexidine

Table 3- Pairwise comparisons of the growth inhibition zones for L. acidophilus caused by different extracts and CHX using the Bonferroni test

Dependent Variable	Group (I)	Group (J)	Mean Difference (I-J)	Sig.
Diameter of growth inhibition zone	AU	AE	4.000000	0.0001
		AM	2.333333	0.004
		ME	-5.000000	0.0001
		CHX	-5.666667	0.0001
	AE	AM	-1.666667	0.061
		ME	-9.166667	0.0001
		CHX	-9.666667	0.0001
	AM	ME	-7.500000	0.0001
		CHX	-8.000000	0.0001
	ME	CHX	-6.000000	0.0001

AU: Aqueous-acidic extract of A nodosum; AE: Ethanolic extract of A. nodosum; AM: Methanolic extract of A. nodosum; ME: Hydroalcoholic extract of P. granatum; CHX: Chlorhexidine

Table 4 summarizes the minimum inhibitory concentrations (MIC) of various extracts against S. mutans and L. acidophilus as determined through broth dilution techniques. Among all tested materials, CHX exhibited the strongest inhibitory effect. Regarding S. mutans, the ethanolic and methanolic extracts of A. nodosum displayed the most notable MIC results. In contrast, for L. acidophilus, the ethanolic extract and aqueous-acidic

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extract of A. nodosum demonstrated superior inhibitory properties.

Table 5 outlines the minimum bactericidal concentration (MBC) of various extracts against S. mutans and L. acidophilus, determined through broth dilution methods. Among the tested substances, CHX demonstrated the highest bactericidal activity. For S. mutans, the aqueous-acidic and methanolic extracts of A. nodosum delivered the most notable results. In contrast, the ethanolic and methanolic extracts of A. nodosum exhibited superior bactericidal effects against L. acidophilus.

Table 4- MIC of extracts against S. mutans and L. acidophilus using the broth dilution methods

Microorganism	Material	Laboratory attempts MIC (g/mL)			MIC (g/mL)
S. mutans	AU	0.1	0.1	0.1	0.1 g/mL
	AE	0.1	0.05	0.1	0.05-0.1 g/mL
	AM	0.1	0.1	0.05	0.05-0.1 g/mL
	ME	0.1	0.1	0.1	0.1 g/ml
	CHX (µg/mL)	10	8	10	8-10 µg/mL
L. acidophilus	AU	0.025	0.0125	0.025	0.0125-0.025 g/mL
	AE	0.0125	0.025	0.025	0.0125-0.025 g/mL
	AM	0.025	0.025	0.025	0.025 g/mL
	ME	0.1	0.1	0.1	0.1 g/mL
	CHX(µg/ml)	2	4	2	2-4 (µg/mL)

Table 5. MBC of the extracts against S. mutans and L. acidophilus using the broth dilution methods

Microorganism	Material	Laboratory attempts MBC (g/mL)			MBC (g/mL)
S. mutans	AU	0.05	0.1	0.1	0.05-0.1 g/ml
	AE	0.1	0.1	0.1	0.1 g/ml
	AM	0.1	0.05	0.1	0.05-0.1 g/mL
	ME	0.1	0.1	0.1	0.1 g/mL
	CHX (µg/ml)	14	12	14	12-14 µg/mL
L. acidophilus	AU	0.1	0.1	0.1	0.1 g/mL
	AE	0.05	0.05	0.25	0.05-0.025 g/mL
	AM	0.05	0.05	0.25	0.05-0.025 g/mL
	ME	0.1	0.05	0.1	0.05-0.1 g/mL
	CHX (µg/mL)	6	4	6	4-6 µg/mL

Using broth dilution methods, the minimum inhibitory concentration (MIC) for S. mutans ranged from 0.05 to 0.1 g/mL for the ethanolic and methanolic extracts of A. nodosum, while the aqueous-acidic extract of A. nodosum and the hydroalcoholic extract of P. granatum demonstrated a MIC of 0.1 g/mL. Regarding minimum bactericidal concentration (MBC), the aqueous-acidic and methanolic extracts of A. nodosum achieved values between 0.05 and 0.1 g/mL, while the ethanolic extract of A. nodosum and hydroalcoholic extract of P. granatum demonstrated an MBC of 0.1 g/mL.

To assess differences in MIC and MBC levels across groups, the Kolmogorov-Smirnov test indicated the lack of normal distribution (P-value < 0.05), prompting the use of the Kruskal-Wallis test. The results revealed statistically significant differences in MIC and MBC values across all bacterial groups (Table 6). For both indices and both bacterial species, significant differences were observed exclusively with the CHX group (Table 7).

Table 6- Test Statistics (Kruskal-Wallis H)

	MIC mutans	MBC mutans	MIC lactobacillus	MBC lactobacillus
Kruskal-Wallis H	10.473	10.473	12.398	12.135
Degree of Freedom	4	4	4	4
P-value	0.033	0.033	0.015	0.016

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Table 7- Multiple Comparisons (Tukey HSD)

Dependent Variable	group (I)	group (J)	Mean Difference (I-J)	P-value
MIC mutans	CH	AU	9.23	<0.001
		AE	9.25	<0.001
		AM	9.25	<0.001
		ME	9.23	<0.001
MBC mutans	CH	AU	13.25	<0.001
		AE	13.23	<0.001
		AM	13.25	<0.001
		ME	13.23	<0.001
MIC lactobacillus	CH	AU	2.64	0.001
		AE	2.64	0.001
		AM	2.64	0.001
		ME	2.56	0.001
MBC lactobacillus	CH	AU	5.23	<0.001
		AE	5.29	<0.001
		AM	5.29	<0.001
		ME	5.25	<0.001

Discussion

This study investigated the antimicrobial effects of various extracts, including ethanolic, methanolic, and aqueous-acidic extracts of Ascophyllum nodosum (A. nodosum) and hydroalcoholic extract of Punica granatum (P. granatum), on Streptococcus mutans (S. mutans) and Lactobacillus acidophilus (L. acidophilus), comparing them with 0.2% chlorhexidine (CHX). The findings demonstrated that while all extracts exhibited antibacterial activity against S. mutans, their efficacy was significantly lower than that of CHX. Among the tested extracts, the methanolic extract of A. nodosum displayed superior antimicrobial effectiveness in pairwise comparisons.

Overall, the ethanolic and methanolic extracts of A. nodosum showed the most effective MIC results, whereas the aqueous-acidic and methanolic extracts of A. nodosum displayed the highest MBC values for S. mutans.

The results from the disc diffusion test demonstrated significant antimicrobial activity of all tested extracts against L. acidophilus), although the effectiveness was notably lower compared to chlorhexidine (CHX). Pairwise comparisons indicated that the hydroalcoholic extract of P. granatum exhibited the highest antibacterial activity against L. acidophilus.

The ethanolic and aqueous-acidic extracts of A. nodosum exhibited superior MIC results, while the ethanolic and methanolic extracts showed higher MBC efficacy against L. acidophilus.

The notable bacteriostatic properties of the aqueous-acidic and ethanolic extracts of A. nodosum, along with the superior bactericidal effects of its ethanolic extract, are primarily attributed to their fucoidan and laminarin content. The antimicrobial mechanism of these compounds involves the binding of glycoprotein receptors on cell surface polysaccharides to the bacterial cell wall, cytoplasmic membrane, and DNA. This interaction disrupts membrane integrity, increasing permeability and causing protein leakage.[20] Research has provided evidence supporting the antimicrobial potential of laminarin-rich extracts.[21]

Fucoidan has gained significant attention for its diverse biological properties, including anti-coagulative, anti-tumor, and antiviral effects.[20] Additionally, it has been shown to enhance the effectiveness of gentamycin and ampicillin when used at one-fourth of their standard dosages, effectively targeting bacterial species within the oral cavity. Both fucoidan and laminarin are also recognized for their use in oral antibiotics to inhibit the growth of Staphylococcus aureus and Escherichia coli.[20] The findings of this study further corroborated the antimicrobial activity of fucoidan in A. nodosum.

Other significant components of A. nodosum include phlorotannins and terpenes, which not only prevent bacterial biofilm formation but also serve as antioxidants, protecting against UV-induced damage. The antimicrobial action of phlorotannins is linked to their ability to disrupt oxidative phosphorylation and interact with bacterial proteins, including enzymes and cell membranes, ultimately causing cell lysis. Moreover, the free fatty acids present in A. nodosum interfere with the electron transport chain and oxidative phosphorylation within bacterial membranes, further contributing to its antimicrobial properties.[20]

The antimicrobial potency of the aqueous-acidic extract of A. nodosum, prepared using the ultrasound-assisted

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extraction method, was found to be superior compared to other extracts. Previous studies have highlighted that this technique enhances the laminarin content in extracts, a key antimicrobial agent [20]. The ultrasound-assisted method is particularly effective for extracting antibacterial compounds from A. nodosum as it avoids high temperatures, toxic solvents, and prolonged extraction times. Additionally, it facilitates the breakdown of the rigid cell wall of A. nodosum.[20]

Research conducted by Jiménez et al. [28]explored the antimicrobial and antioxidant effects of the acetone extract of A. nodosum against a variety of Gram-positive and Gram-negative bacteria using MIC assays. Their findings indicated moderate antimicrobial activity of the acetone extract, particularly against Gram-positive bacteria. However, their study focused solely on the acetone extract, whereas this research examined aqueous-acidic, ethanolic, and methanolic extracts to provide a broader understanding of antimicrobial efficacy. The MIC for the acetone extract ranged between 0.2 to 0.5 g/mL in their study, while the MIC values for the other extract types in this study ranged from 0.05 to 0.1 g/mL. These findings suggest that the acetone extract may possess weaker antimicrobial properties compared to the other extract types. The enhanced efficacy of the aqueous-acidic, ethanolic, and methanolic extracts can likely be attributed to their higher phlorotannin concentrations.

Mattsson and Wikner [14] studied the impact of daily consumption of ProDen PlaqueOff® tablets, containing A. nodosum, on the formation of dental plaque and calculus. Their findings indicated a reduction in plaque and calculus accumulation in adults. Conducted as an in vivo study, that research confirmed the antimicrobial properties of A. nodosum. Wikner et al. [14] explored the systemic effects of A. nodosum tablets on supragingival plaque and calculus. They observed a dose-dependent decrease in plaque accumulation after several weeks of daily tablet use.

Reddy et al. [29] investigated the antibacterial, antioxidative, and antimalarial properties of P. granatum. Their results, aligning with the findings of this study, attributed the antimicrobial efficacy of P. granatum to its tannin, ellagitannin, and phenolic acid content. The pomegranate fruit contains compounds such as ellagic acid, punicalin, punicalagin, and tannin, which possess antioxidative, antiplasmodial, and antibacterial properties. Naz et al. [30]evaluated the methanolic extract of pomegranate peel, demonstrating its effectiveness against various bacterial species, including Corynebacterium, Staphylococcus, Streptococcus, Bacillus subtilis, Shigella, Salmonella, Vibrio cholerae, and Escherichia coli. This extract exhibited stronger antibacterial activity against Gram-positive bacteria. Among its purified constituents, gallic acid displayed the highest antimicrobial efficacy due to its phenolic structure. Variability in results across studies may stem from differences in solvent types and extraction methods.

Vasconcelos et al. [31] examined the antimicrobial activity of P. granatum Linn. gel in comparison to miconazole against S. mutans, Streptococcus sanguinis, and Streptococcus mitis. The study highlighted the superior ability of pomegranate gel to inhibit microbial adhesion to surfaces in the presence of 5% sucrose when compared to miconazole. It is noteworthy that gel formulations, with their higher consistency, often demonstrate greater efficacy than liquid extracts. Furthermore, a review article [32]reported the beneficial effects of pomegranate fruit on periodontal health, including enhanced fibroblast migration, collagen synthesis, and angiogenesis. Methanolic ointments derived from pomegranate peel (5%, 10%, and 15%) were shown to promote wound healing, a property linked to the tannin and polyphenol content. That study emphasized both the healing and antimicrobial capabilities of P. granatum.

This study encountered certain limitations, particularly the lack of isolation and separate evaluation of the extracts' constituents. Isolating these individual components could have helped identify their maximum antibacterial potential while minimizing potential complications. Future research should prioritize isolating and analyzing the specific constituents of A. nodosum and P. granatum. Additionally, the antimicrobial efficacy of these extracts should be tested against a broader spectrum of oral pathogens, with a specific emphasis on Gram-negative bacteria.

Moreover, it is essential to assess the cytotoxicity of these extracts on human oral fibroblasts through in vivo studies before their integration into oral care products such as mouthwashes, toothpaste, or chewing gums.

Conclusion

This study demonstrated the promising antibacterial effects of various extracts derived from Ascophyllum nodosum and Punica granatum on Streptococcus mutans and Lactobacillus acidophilus, two significant contributors to dental caries and oral biofilm formation. While chlorhexidine (CHX) exhibited superior antimicrobial activity overall, the extracts—particularly the aqueous-acidic, ethanolic, and methanolic extracts of A. nodosum and the hydroalcoholic extract of P. granatum—showed considerable inhibitory and bactericidal effects against

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both bacterial strains. These findings underscore the potential of these natural compounds to serve as alternative antimicrobial agents, especially for individuals who experience adverse reactions to CHX.

Furthermore, the ultrasound-assisted extraction technique was highlighted as an effective method for optimizing the antibacterial potency of A. nodosum extracts.

Acknowledgement: None.

Author Contributions:
M.L. and B.F.: Methodology, Writing – Original Draft, P.A.: Project Administration, Writing – Review & Editing, S.T.H.: Visualization, Supervision, F.E.: Methodology, Resources, E.M.: Data Curation, Formal Analysis.

Funding: No funding was obtained for the current study.

Ethical Approval Code: This study was approved by the Ethics Committee of the Islamic Azad University, Dental School, Tehran, Iran (Approval No: D/159/P, Date: March 11, 2022).

Informed Consent Statement: None.

Data Availability Statement: None.

Conflict of Interest: No conflicts of interest to declare.

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