

Article

Ulvan as a Reducing Agent for the Green Synthesis of Silver Nanoparticles: A Novel Mouthwash

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Abstract: The antibacterial activity of an Ulvan-based silver nanoparticle (AgNP) system was evaluated in the current study. The green synthesis of biogenic silver nanoparticles was conducted using Ulvan, a sulphated polysaccharide extracted from *Ulva lactuca*. A novel mouthwash containing AgNPs was prepared, and tested for its efficacy and safety. AgNPs were confirmed with spectrophotometric analysis (UV–A visible spectrophotometer), and the characterisation was established with Fourier transform infrared spectroscopy (FTIR), X-ray diffraction (XRD), and transmission electron microscopy (TEM). The AgNPs were spherical, and their average size was 8–33 nm, as shown via TEM. The antioxidant assay was conducted via DDPH assay, wherein the AgNPs, at a concentration of 50 µL/mL, showed 93.15% inhibition. Furthermore, anticancer activity was tested by evaluating the cell viability utilising the method of an MTT assay on the 3T3-L1 cell lines. AgNPs, at 30 µL/mL, showed maximal cell viability, denoting no cytotoxic effect. The silver-nanoparticle-based mouthrinse, at a concentration of 100 µL/mL, demonstrated antimicrobial activity against *Streptococcus mutans*, *Staphylococcus aureus*, *Lactobacillus*, and *Candida albicans*. This study shows that mouthwash prepared from the Ulvan-silver nanoparticle system could be a nontoxic and effective oral antimicrobial agent.

Keywords: anticarcinogenic agents; biocompatible materials; dental caries; dentistry; nanoparticles; polysaccharides



Citation: Mohandoss, S.; Murugaboopathy, V.; Haricharan, P.B.; Hebbal, M.I.; Saadaldin, S.; Soliman, M.; Eldwakhly, E. Ulvan as a Reducing Agent for the Green Synthesis of Silver Nanoparticles: A Novel Mouthwash. *Inorganics* **2023**, *11*, 5. <https://doi.org/10.3390/inorganics11010005>

Academic Editors: Kenneth J.D. MacKenzie and Navid Rabiee

Received: 25 October 2022

Revised: 11 December 2022

Accepted: 18 December 2022

Published: 22 December 2022



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1. Introduction

Nanotechnology is a new topic in science, with an array of applications in the field of medicine. Nanoparticles (0.1–100 nm) possess unique physical and chemical properties compared to macromolecules. The biological synthesis of nanoparticles, as a nontoxic and ecofriendly process, had attracted researchers. Nanoparticles, owing to their larger surface area, provide better interactions with microbial agents. Nanoparticles can also effectively penetrate bacterial cells and exert bactericidal activity better than traditional macro-/microparticles can [1]. Given these advantages, nanoparticle systems can be effective pharmaceutical agents.

The biological properties of nanoparticles depend on their size, shape, and morphology, which may be influenced by the method of synthesis [2]. Nanoparticles are synthesised via chemical, physical, and biological methods [3]. Chemical methods require reducing and stabilising agents, and physical methods include evaporation–condensation and laser

ablation [4]. Biogenic synthesis, on the other hand, is more environmentally friendly, and employs the usage of plants, fungi, microorganisms, and algae as reducing agents [5].

Silver nanoparticles (AgNPs) are the most important nanomaterials among metallic nanoparticles used in biomedical preparations. They have multifunctional bioapplications, such as in the production of antibacterial, antifungal, antiviral, antiangiogenic, anti-inflammatory, and anticancer agents. They are extensively used as therapeutic agents due to the targeted delivery of nanodrugs and their stability [6]. The extensive application of silver nanoparticles as anticancer agents is also being explored. Silver nanoparticles augment autophagy, and could potentially be used for the treatment of cancer and inflammatory disorders. Seaweed, with its active secondary metabolites, is a potential source of antimicrobial substances. Sujatha L et al. demonstrated that the crude extracts of *Ulva lactuca* have antimicrobial activity against pathogens [7]. The main biological agent that is responsible for antimicrobial activity is Ulvan, a sulphated polysaccharide of *Ulva* spp. There are also no associated toxic effects reported with the use of Ulvan [8].

The most common infectious diseases of the oral cavity are dental caries and periodontal disease. Plaque biofilm is a microbial consortium with an exopolysaccharide matrix associated with these oral diseases. This complex structure provides nutrients and protection from adverse environmental factors, including disinfectants and antibiotics. The removal and control of plaque biofilms require active biological agents penetrating the microbial biofilm [9]. Many intensive efforts have been put forward by various researchers into formulating an effective antiplaque agent. However, owing to toxicity or poor efficacy, these attempts were not met with success [10]. Currently available antiplaque agents are chemical-based, with constituents such as triclosan, chlorhexidine, and gluconate. The long-term use of chemical antiplaque agents has shown various side effects, such as taste alterations and allergic stomatitis. This underlines the importance of exploring natural and biocompatible alternatives that are both safe and effective [11].

Previous studies showed that polysaccharides are excellent candidates for the green synthesis of silver nanoparticles. They are known for their low toxicity, ideal molecular structure, and excellent biocompatibility [12,13]. We hypothesised that Ulvan polysaccharide-mediated biologically synthesised nanosilver particles may be effective against plaque microbiota with no evident toxicity. Hence, this in vitro study was conducted to test the antimicrobial efficacy of a mouthrinse prepared from silver nanoparticles mediated by Ulvan polysaccharides against oral microbes such as *S. mutans*, *S. aureus*, *Lactobacillus*, and *C. Albicans*. Moreover, the cytotoxic effect of AgNPs was assessed using an MTT assay.

2. Materials and Methods

2.1. Ulvan Extraction from *Ulva lactuca*

The current study extends the findings of a previous study that tested the antimicrobial activity of selenium nanoparticles synthesised using *Ulva lactuca* [14]. The green marine alga *Ulva lactuca* was collected from the Gulf of Mannar Biosphere in Mandapam, Rameswaram, India. The collected algae were cleaned using water, dried in the shade, and later stored at room temperature. Pengzhan et al. [15] demonstrated the method for polysaccharide Ulvan extraction that was employed here as well. The mean yield was $38.3 \pm 1.2\%$ ($n = 6$) (38.3 g of Ulvan/100 g of biomass). The Ulvan extract (1%) was prepared by adding 10 g of prepared Ulvan powder (the method or phase of powdering the algae is not mentioned) in 100 mL of double-distilled water for about 10 min at 70 °C. Whatman number 1 filter paper was employed to filter the solution after boiling, and the obtained filtrate was further used to prepare the nanoparticle system.

2.2. Green Synthesis of Biogenic Silver Nanoparticles

We mixed 1 mL of the Ulvan extract filtrate with 10 mL of 30 mM AgNO₃ aqueous solution. This prepared solution was then placed in an incubator-cum-shaker at 250 rpm until there was a change in colour from pale yellow to yellowish brown, confirming the nanoparticle system formation. The AgNPs were confirmed using a UV-visible spectropho-

tometer (model UV-D3200) at different time intervals of 1, 12, 18, 24, 48, and 72 h, followed by centrifuging the solution at 10,000 rpm for 30 min. The pellet obtained after centrifuging was washed with double-distilled water followed by absolute ethanol, and then dried in a hot air oven at 80 °C for 2 h. This prepared pellet was stored in air-tight containers for further analysis.

2.3. Characterisation of AgNPs

The characteristic feature of reducing metal salts to nanoparticles is the change in colour, which was visualised by the colour transforming into yellowish brown. The synthesis of AgNPs was confirmed using UV-vis spectrophotometric analysis. We analysed 2 ml aliquots of the prepared solution using a Shimadzu 1700 UV-Vis spectrophotometer (UV-vis; Optizen POP; Mecasys, Daejeon, Korea) with wavelengths ranging from 200 to 650 nm using a scanning speed of 1856 nm/min. The readings were recorded at 1, 12, 18, 24, 48, and 72 h intervals. The crystal density, phase composition, and size of the synthesised NPs were assessed with an X-ray diffractometer (PAN analytical X-Pert PRO), (Bruker, Bremen, Germany), operating at 30 kV and 40 mA employing CuK α radiation with about 1.54060 Å. The surface morphology of the AgNPs and sizes were estimated with the use of a 200 kV transmission electron microscope (JEOL, Tokyo, Japan). FT-IR analysis was performed using the KBr pellet method at 4 cm⁻¹ (Shimadzu model 400, (Spectrum One System, Perkin-Elmer, Waltham, MA)) to identify the biological compounds responsible for the formation and the stability of AgNPs.

2.4. Antioxidant Activity—DPPH Radical Assay

The DPPH free radical scavenging activity of AgNPs was determined with the method suggested by Qidwai et al. [16]. Typically, different concentrations (10–50 µg/mL) of nanoparticles are mixed with 1 mL of 0.1 mM DPPH in methanol solution and 450 µL of 50 mM Tris-HCl buffer (pH 7.4), and incubated for 30 min. After incubation, a reduction in the number of DPPH free radicals was measured on the basis of absorbance at 517 nm. Ascorbic acid was used as the standard control. The percent inhibition was calculated from the following equation: % inhibition = [absorbance of control – absorbance of test sample/absorbance of control] × 100.

2.5. Cell Viability Assay

The cell viability was assessed using the MTT assay method described by Merlo et al. [17] using a 3T3-L1 cell line. For high-throughput screening, the cells were placed separately in 96-well plates at a concentration of 1×10^5 cells /well. The cells were washed twice with 100 µL of serum-free medium and starved at 37 °C for 60 min. After starvation, they were treated with the Ulvan AgNPs for 24 h. After the treatment period, the medium was aspirated, and serum-free medium containing MTT (5 mg/mL) was added and incubated for 4 h in a CO₂ incubator at 37 °C. Then, the cells were washed with 200 µL of Dulbecco's phosphate-buffered saline (DPBS) at pH 7.4, followed by the addition of 100 µL of DMSO solution to dissolve the crystals. A Robotnik ELISA analyser was used to read the spectrophotometrical absorbance of formazan dye. The ideal wavelength for the absorbance of the solubilised crystal of formazan was 570 nm, which was determined using Graphpad Prim5 software.

2.6. Preparation of Mouthrinse

The Ulvan-based silver nanoparticle-based mouthrinse was prepared using the following method: 10 mL of distilled water was poured into a test tube, 900 µL of AgNPs was added to this tube, and 0.4 ml of thymol and 0.16 gm of sodium stearyl lactylate were added.

2.7. Antimicrobial Activity of Ulvan-Mediated AgNP Mouthrinse against Oral Pathogens

Institutional ethical clearance (Letter no. ECR/290/Indt/PY/2018) was obtained before the start of the study. The antibacterial activity of AgNPs at different concentrations

was assessed with the agar well diffusion method. Microorganisms *Streptococcus mutans* (MTCC 497), *Lactobacillus* (MTCC 10307), *Candida albicans* (MTCC 3017), and *Staphylococcus aureus* (MTCC 3103) were procured from the Institute of Microbial Technology, Chandigarh, India. Using a sterile spreader, secondary cultures of microbial suspension were dispersed uniformly over the surface of Muller Hinton agar and rose Bengal agar plates. Different mouthrinse concentrations with 25 μ L (2.5 mg of AgNPs), 50 μ L (5 mg of AgNPs), and 100 μ L (10 mg of AgNPs) of nanoparticles were aspirated via a sterile micropipette, and placed onto the wells created in the agar plate. These plates were then incubated at 37 °C for 24 to 48 h. Commercial antibiotic ampicillin (50 mg/mL) was used for positive control, and the zone of inhibition (mm) was measured for each plate as compared with the control.

3. Results

3.1. Characterisation of AgNPs

3.1.1. UV–Vis Spectra Analysis

The UV–vis spectrophotometer is documented to be the most appropriate tool [18] for assessing the optical properties and for the confirmation of the synthesis of nanoparticles. Figure 1 shows the changes in the absorption band in the 350–500 nm spectrum, and it slowly decreased from 1.3 to 0.8, which was indicative of nanoparticle reduction. Spectral analysis showed the maximal peak at 450 nm after 72 h of observation. After 48 h, the colour change was initiated, and it became evident at 72 h, which also concurred with the peak found in the UV spectral reading at 450 nm (Figure 1).

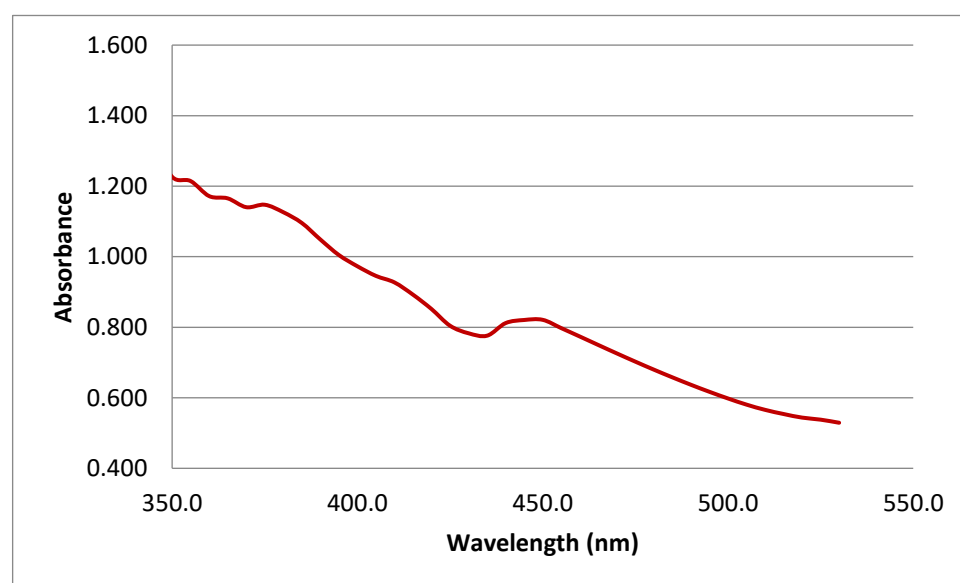


Figure 1. UV–vis absorption spectrum of AgNPs produced by the Ulvan from *Ulva lactuca*.

3.1.2. Transmission Electron Microscopy and X-ray Diffraction Analysis

The surface morphology assessment of the AgNPs performed using TEM revealed spherical structures with an average diameter in the range of 8–23 nm with a clear background (Figure 2).

The XRD analysis shown in Figure 3 confirmed the crystalline phase of the synthesised AgNPs. The 2θ values of the XRD pattern with Bragg's angles 39.92°, 43.12°, 64.23°, and 78.12° correspond to 111 of the face-centred cubic structure of silver. The silver nanoparticles were 8 nm, estimated with Scherrer's equation by determining the width of the (111) Bragg reflection. XRD also revealed some background noise that could have been produced by the formation of silver nanoparticles reduced by the bioactive compounds.

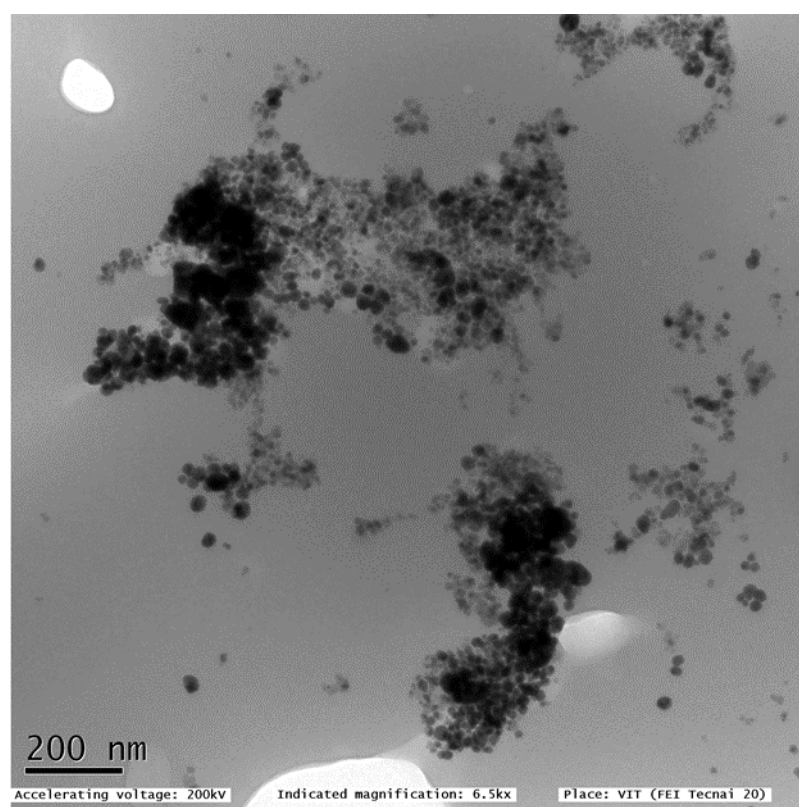


Figure 2. Transmission electron microscopy (TEM) micrograph of AgNPs produced with Ulvan from *Ulva lactuca*.

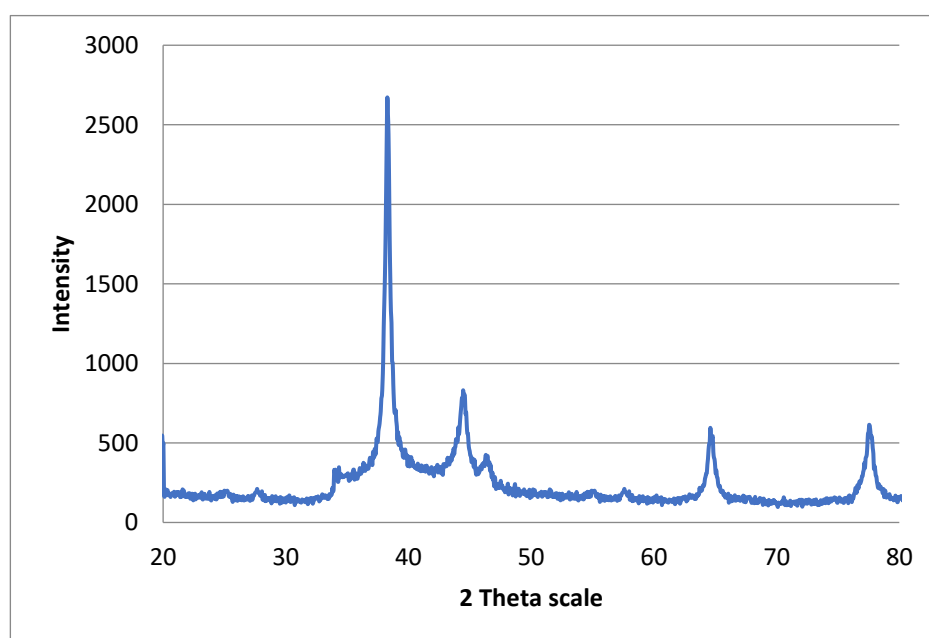


Figure 3. X-ray diffraction (XRD) spectra of AgNPs produced with Ulvan from *Ulva lactuca*.

3.1.3. FT-IR Assessment

Fourier transform infrared (FT-IR) was used to assess and identify the chemical groups present in AgNPs, and chemical groups responsible for the formation of nanoparticles and their stability. The FTIR analysis showed some shifting of the peaks; peak intensity decreased/increased and disappeared, as shown in Figure 4. Figure 4 illustrates the peaks at

1094 cm^{-1} corresponding to C–O stretch ethers. The appearance of the peak represents the carboxylate and carbon in the aromatic groups of terpenoid and saponins. The 1386 cm^{-1} to N=O bend nitro groups, 1621 cm^{-1} to C=O stretch amides, and 3387 cm^{-1} represent N–H stretch secondary amines, confirming the glucose structure attached to the terpenoid saponin as aldehyde that oxidises into gluconic acid. These peaks confirmed that the extract occurred at the carboxylate groups and the stretching of esters. Further, the sharp band of the brag peak confirmed the stabilisation of the synthesised AgNPs, as shown Figure 5. The peak changes support the impact of the functional groups of Ulvan as reducing and stabilising agents for silver nanoparticles.

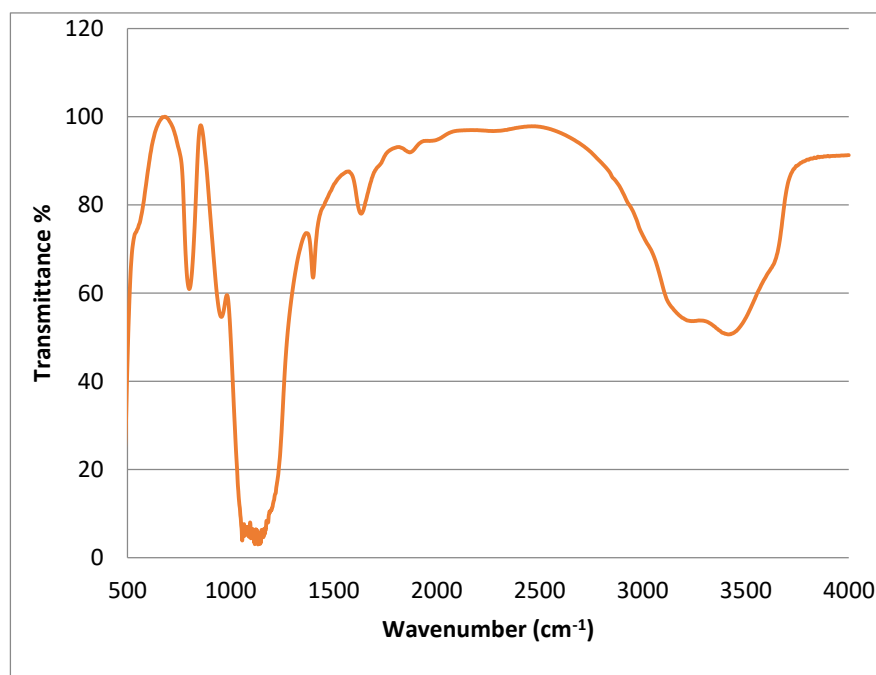


Figure 4. Fourier transform infrared (FTIR) spectroscopy of AgNPs produced with Ulvan from *Ulva lactuca*.

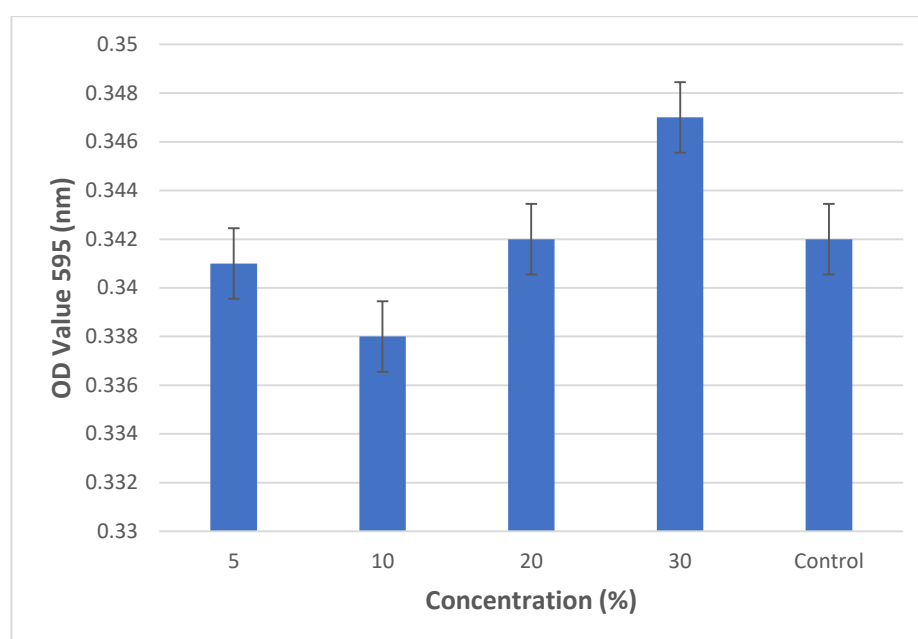


Figure 5. Cell viability assay after treatment with AgNPs for 24 h compared to control.

3.2. Cell Viability Assay

The cytotoxic effect of AgNPs was assessed using an MTT assay. Cell viability on the 3T3 L1 cell line at all concentrations (5%, 10%, 20%, and 30%) is depicted in Figure 5.

3.3. Antioxidant Activity

The excellent antioxidant properties of AgNPs were confirmed using a DDPH assay. Ascorbic acid was used as the control, and AgNPs confirmed antioxidant properties at all concentrations (Figure 6). The antioxidant efficacy increased with the increasing dose; at a concentration of 50 $\mu\text{L}/\text{mL}$, it demonstrated a 93.15% inhibition.

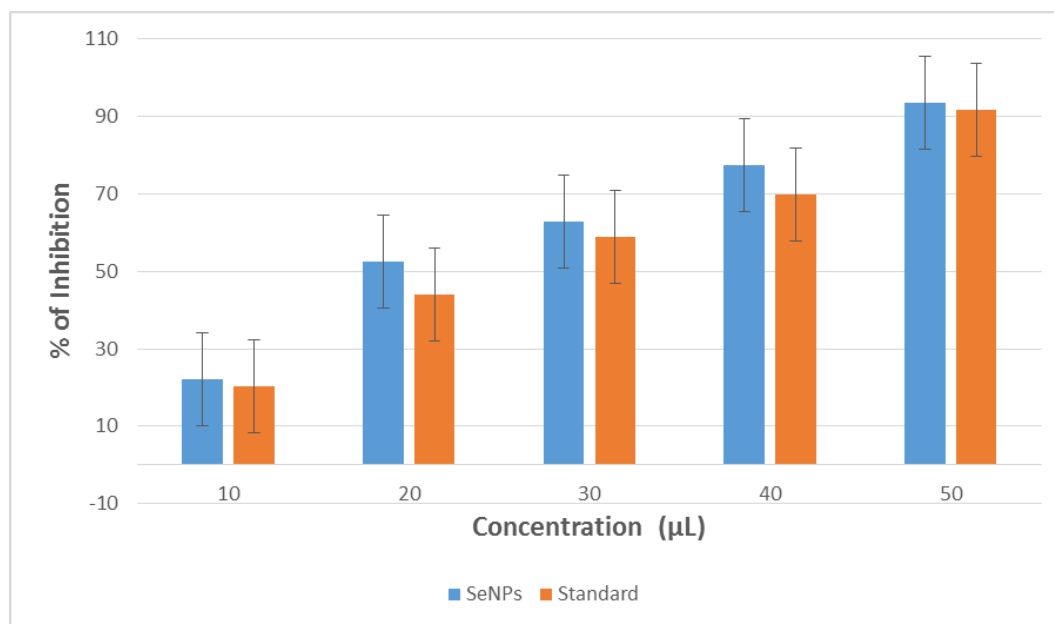


Figure 6. DDPH antioxidant assay of AgNPs produced with Ulvan from *Ulva lactuca*.

3.4. Antimicrobial Activity against Oral Pathogens

The antimicrobial efficacy of different concentrations of am Ulvan-mediated AgNP mouthrinse is presented in Figure 7. The mean zone of inhibition (ZOI) of biogenic AgNPs against oral pathogens is presented in Figure 8. For *S. aureus*, the mean ZOI was 11 mm at 25 μL concentrations of AgNPs, which was less than that of the control. The concentrations of AgNPs at 50 μL were 13 mm, and at 100 μL , they were 15 mm, almost equivalent to the control antibiotic. Similarly, for *S. mutans*, an AgNP concentration of 25 μL was less effective, with a mean ZOI at 10 mm, whereas at 50 and 100 μL concentrations, the ZOI was 13 and 15 mm, respectively, as compared to the control (17 mm).

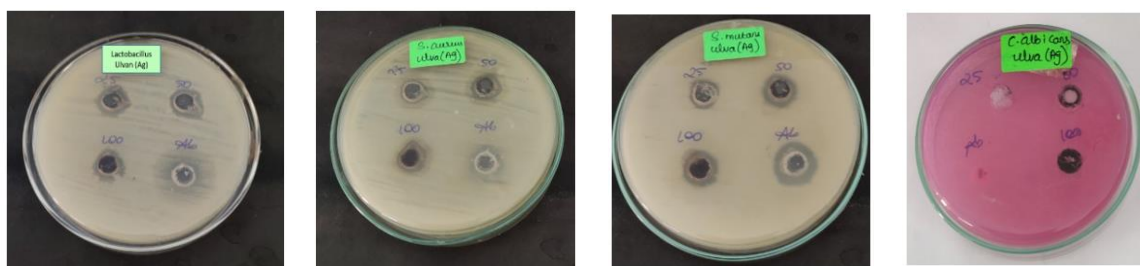


Figure 7. Antimicrobial activity of AgNPs produced with Ulvan from *Ulva lactuca* (*Lactobacillus*, *S. aureus*, *S. mutans*, and *C. albicans*).

Antibacterial efficacy against *Lactobacillus* at concentrations of 25 and 100 μL with a ZOI of 15 mm was close to the standard (18 mm). Antifungal efficacy was relatively

moderate at all concentrations with a mean ZOI (10, 11, and 12 mm) compared to the control, i.e., amphotericin B (20 mm).

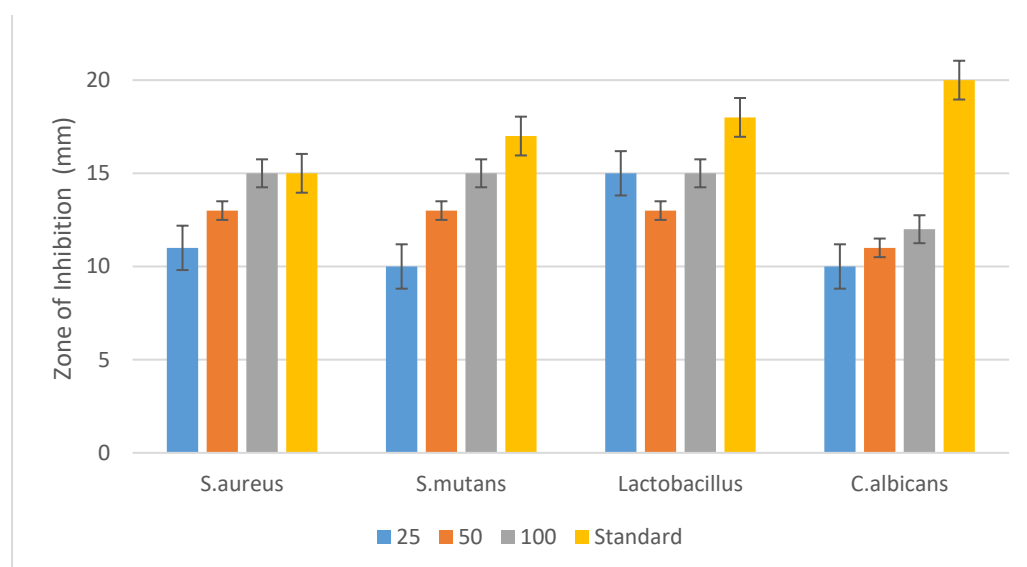


Figure 8. Antimicrobial activity of Ulvan AgNP mouthrinse against oral pathogens.

4. Discussion

Ulva lactuca, the green macroalga, contains many phytochemicals such as alkaloids, flavonoids, phenols, xanthoproteins, and terpenoids that have antibacterial and antioxidant properties, rendering it ideal for the current study. Biogenic silver nanoparticle synthesis was performed using Ulvan extracted from *Ulva lactuca*, which functioned as a reducing agent.

The synthesis of AgNPs in this study was confirmed with the visual observation of mild colour change and the UV–vis spectral findings. A similar absorbance spectrum in the 420–460 nm range was observed for AgNPs synthesised using *Ulva* spp. [19].

A characterisation study was conducted to confirm the AgNPs formation. The TEM revealed that the spherical structures obtained here were similar to the spherical structures with a diameter of 5–15 nm observed during the previously performed synthesis of AgNPs using brown macroalga *Sargassum muticum* [20].

X-ray diffractometer analysis was performed to assess the crystalline structure of the silver nanoparticles. The crystalline phase revealed with XRD analysis was similar to that of AgNPs synthesised from *Acanthophora specifera* [20].

Cell viability assay was conducted to test the cytotoxicity of the reduced silver nanoparticles. In the current study, the excellent cell viability on the 3T3 L1 cell line at all observed concentrations agreed with previous studies showing that AgNPs possess anticancer activity [21,22]. The cell viability assay showed that the AgNPs had no toxicity, even at higher threshold of concentrations, proving their potential safety. The phytochemicals flavonoids and phenolic compounds are responsible for the antioxidant capacity of AgNPs [23].

Silver nanoparticles can act on bacterial cells by signalling the transduction systems that can monitor fluctuating environmental and intracellular conditions. These mechanisms mediate the adaptive responses to the prevailing conditions. Previous studies showed that AgNPs of smaller particle size had better antibacterial effects owing to the release of more Ag⁺ ions. Additionally, this was effective against all serotypes of *S. mutans* [23,24]. The AgNP-mediated mouthrinse possessed superior antimicrobial efficacy than that of commercial control ampicillin, even though the concentration of AgNPs was only minimal at 2.5, 5, and 10 mg, as equated to the dose of 50 mg of the control antibiotics. This finding was concurrent with that of the study by Panpaliya et al. [25], who reported that AgNPs had better antimicrobial efficacy at a lower concentration. Another study, by Gómora et al. [26],

reported the antibacterial activity of biosynthesised AgNPs against oral pathogens. They discussed that AgNPs could inhibit the enzymes of the respiratory cell cycle and damage DNA synthesis, leading to microbial cell death. AgNPs showed excellent antibacterial efficacy against *S. aureus* and *E. coli*, as supported by the findings of Alnairat et al. [27]. The antibacterial mechanism and antibiofilm properties of AgNPs were well-documented by Qing et al. [28], and the study showed that a mouthrinse based on AgNPs is effective against microbes of the dental plaque biofilm.

Fungal infections are also a cause of concern for humankind owing to their role in many primary and secondary infections. Oral thrush is one of the major fungal diseases of the oral cavity. Due to their evolving resistance mechanisms, the efficacy of routine antifungal agents is limited. Therefore, silver nanoparticles with proven antibacterial activity may also be useful against fungal infections. Thus, the biogenic synthesis of silver nanoparticles has been promising in providing excellent drug delivery and stability against fungal infections. However, in the current study, there was a moderate effect of silver nanoparticles against *Candida albicans* [29,30].

Extensive research was carried out to test the application of silver nanoparticles in treating dental diseases. A detailed review by Fernandez et al. [31] discussed the potential of silver nanoparticles and their antibacterial activity against *Streptococcus mutans*, *Lactobacillus*, and *Staphylococcus aureus*. The efficiency of AgNPs increased when the size of the nanoparticles was reduced, thus supporting the findings of the current study. In addition, the efficacy of AgNPs was improved when a green synthesis methodology was followed for the formation of nanoparticles [31], which was adopted here.

The antimicrobial characteristics of AgNPs were thoroughly reviewed and reported by Yazdanian et al. [32]. That review encompassed various applications, and concurs with the hypothesis and findings of the current study. Thus, the current study adds value to the research findings that the green synthesis of AgNPs utilising the biologically active components of marine algae could synergistically produce an effective antimicrobial product that can be used to prevent plaque-induced oral diseases.

In addition to antimicrobial activity, this study also showed that the biosynthesised silver nanoparticles had anticancer activity, as evidenced via an antioxidant assay. Previous studies also showed anticancer activity [33,34].

The limitation of the current study is the in vitro design, which renders no direct link to the therapeutic applications or pharmacokinetics of the implied drug. Understanding its absorption mechanisms, biodistribution, and metabolism is essential to determine its toxicity and clinical effectiveness. However, limited studies have shown that AgNPs are less toxic when green synthesis is followed, and this necessitates studies towards testing its clinical applications. Overall, the findings of the current study suggest that AgNPs could emerge as a potential antimicrobial agent for use in the control of pathogenic bacteria, having an extended implication in lowering caries activity and in the prevention of periodontal infections.

5. Conclusions

In this study, a biogenic silver nanoparticle mouthrinse was prepared using the Ulvan polysaccharide extracted from *Ulva lactuca*. UV-vis spectroscopy and transmission electron microscopy were used to study the characteristic traits of silver nanoparticles. The average size of the nanoparticles was 8–33 nm. The AgNPs also exhibited excellent antioxidant properties, and a cell line viability study showed that the AgNPs had no toxicity at all concentrations. The antimicrobial effectiveness of the AgNP-based mouthrinse was assessed against *Lactobacillus*, *S. mutans*, and *S. aureus*. This study showed that Ulvan, a polysaccharide-based mouthrinse, can be used as an antimicrobial agent for common oral diseases. However, extensive research in this field is required for the benefit of humanity.

A mouthrinse produced from Ulvan polysaccharide (*Ulva lactuca*) and augmented with silver nanoparticles (8–33 nm) demonstrated excellent antimicrobial effectiveness against *Lactobacillus*, *S. mutans*, and *S. aureus*, with no cell toxicity. Thus, the AgNP-based

mouthwash may be implicated in both preventive and therapeutic roles for the management of major oral diseases, namely, dental caries and periodontitis.

Author Contributions: Conceptualisation, S.M.; methodology, V.M.; software, P.B.H.; validation, M.H.; formal analysis: S.S.; investigation, M.S.; resources, E.E.; data curation, S.M.; writing—original draft preparation, V.M.; writing—review and editing, P.B.H.; visualisation, M.I.H.; supervision: S.S.; project administration, M.S.; funding acquisition, E.E. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Princess Nourah bint Abdulrahman University Researchers Supporting Project (number PNURSP2022R98), Princess Nourah bint Abdulrahman University, Riyadh, Saudi Arabia.

Institutional Review Board Statement: Not applicable. Due to the material-based in vitro nature of the study and that it did not include any human or animal subjects or tissues, it was exempted from the IRB ethical approval.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

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