Antimicrobial, antioxidant and cytotoxic activities of silver nanoparticles synthesised using green and brown algae

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Abstract

Surgical site infections are common, occurring in 2.3% of all surgeries, resulting in increased hospital stays and extra medical costs. Silver nanoparticles are being explored as a possible antibiotic prophylaxis, but current methods of synthesis make use of toxic chemicals and have a high climate change potential. The project explored the use of the aqueous extract of *Ulva* and *Sargassum* in the eco-synthesis of silver nanoparticles that are suitable for use as an antibacterial prophylaxis. Silver nanoparticles were synthesized by mixing the aqueous extracts of *Ulva* and *Sargassum* with aqueous silver nitrate. The presence of silver nanoparticles was confirmed using UV-vis spectrophotometry, and the nanoparticles were characterized using scanning electron microscopy. The nanoparticles' antibacterial properties were tested using the colony count test with *E. coli* and *S. epidermidis*, the cytotoxic properties with *C. elegans*, and the antioxidant properties with DPPH. The peak wavelengths of both the *Ulva* and *Sargassum* solutions around 420nm indicated the presence of silver nanoparticles, while scanning electron microscopy showed that the nanoparticles were mainly between 30nm and 50nm in diameter. There was a significant decrease in the number of colony forming units, absorbance of DPPH, and *C. elegans* survival rate for the setups with the synthesized nanoparticles ($p<0.05$) as compared to the control. The free radical scavenging activity was 58% for *Ulva*, and 20% for *Sargassum* synthesized nanoparticles. The research showed that the aqueous extract of *Ulva* and *Sargassum* could synthesize silver nanoparticles that had antibacterial, antioxidant, but cytotoxic properties. Thus, *Ulva* and Sargassum extracts are effective, safe, and eco-friendly alternatives to conventional chemical methods of synthesis of silver nanoparticles. The synthesised silver nanoparticles were however unsuitable for use as a prophylaxis, but could have applications in the biomedical field to kill cancer cells.

Introduction

Surgical site infections are a common complication of surgery, appearing in 2.3% of all surgeries in Singapore, resulting in increased hospital stays and medical costs, putting strain on the healthcare system (Cai et al., 2017). Currently, antibiotic prophylaxes are given to reduce the risk of surgical site infections. While they have been shown to reduce surgical site infection rates, there has been a decline in its efficacy due to the rise of antibiotic resistant bacteria (Gandra, Trett, Alvarez-Uria, Solomkin, & Laxminarayan, 2019).

Silver nanoparticles are being explored as an alternative to antibiotic prophylaxis as they displayed strong antibacterial properties against many Gram-positive and Gram-negative bacteria, even at low concentrations of 4 $\mu g m\ell^{-1}$ (Rai, Deshmukh, Ingle, & Gade, 2012). There are many mechanisms of action for the antibacterial properties of silver nanoparticles. Silver nanoparticles damage intracellular structures like the mitochondria and ribosomes, inhibiting normal function, and catalyse the production of reactive oxygen species, leading to oxidative stress and induced cellular toxicity (Ivanova et al., 2018; Kedziora et al., 2018).

Currently, silver nanoparticles are mainly produced using chemical methods which generally involve the reduction of Ag^+ ions into Ag, using chemical reducing agents, such as sodium borohydride and sodium citrate. The individual silver atoms then agglomerate, forming the nanoparticles. (Ivanova et al., 2018). However, many of the chemicals used are hazardous to the environment and carry a higher climate change potential (LewisOscar et al., 2016).

With demand for silver nanoparticles expected to increase, there is a growing need for an eco-friendly, yet cost effective way to produce silver nanoparticles. Plant mediated synthesis of silver nanoparticles has sparked interest in the scientific community, presenting a potential eco-friendly, simple, and affordable solution to the growing demand of silver nanoparticles. Algal extracts, like those of *Ulva* and *Sargassum*, have a rich phytochemical composition, containing phenols, flavonoids, catechins, and tannins that can help reduce silver ions into silver atoms. They also contain capping and stabilising agents like glutamic acid that can help maintain the size and shape of the nanoparticles (Ivanova et al., 2018; Dominguez & Loret, 2019). The use of *Ulva* and *Sargassum,* in synthesising silver nanoparticles is even more attractive due to their abundance along the reef flats of shores of southern Singapore, with 54% of the southern reef

flats being covered by *Sargassum* (Low & Chou, 2013). In fact, they are often regarded as pests and discarded. Thus, the use of algae extracts in the ecosynthesis can help better make use of resources and reduce wastage.

Plant mediated synthesis also results in greater surface functionalization of the silver nanoparticles. Phenolic acids, polyphenols, flavonoids, and glycosides have a strong affinity to the silver nanoparticles, helping to enhance the nanoparticles' antibacterial and antifungal properties. The compounds also provide antioxidant properties (Ivanova et al., 2018), potentially mitigating the risk of cytotoxicity to healthy human cells from reactive oxygen species, making these nanoparticles suitable as a prophylaxis for surgery.

This project aimed to explore the potential of using the aqueous extract of *Ulva* and *Sargassum* as an eco-friendly and cost effective method of synthesising silver nanoparticles, that can be used as an antibacterial prophylaxis to prevent surgical site infections.

1. Objectives and hypotheses

Objectives

The objectives of the project were to investigate the ability of *Ulva* and *Sargassum* extracts to synthesise silver nanoparticles, as well as the physical, antibacterial, antioxidant, and cytotoxic properties of the synthesised silver nanoparticles.

Hypotheses

We hypothesised that *Ulva* and *Sargassum* extracts were able to synthesise silver nanoparticles that show antioxidant properties, are antibacterial towards the Gram-positive *Staphylococcus epidermidis* and Gram-negative *Escherichia coli* bacteria, and are cytotoxic towards the nematode *Caenorhabditis elegans*.

2. Methods and Materials

Procedures

Preparation of algae extract

Ulva and *Sargassum* was purchased from Algae Barn (5900 E 58th Ave, Commerce City, CO 80022, USA) and dried under the sun for 2 hours.10 grams of dried *Ulva* or *Sargassum* was added to 100 mℓ of deionised water and mixed with a magnetic stirrer for 2 hours. The mixture was then sonicated at low frequency for 15 minutes to free up trapped biomolecules in the cells, and filtered through a filter paper. The filtrate (algae extract) was then collected and stored at 4°C until needed.

Synthesis of silver nanoparticles using algae extracts

0.05mℓ of algae extract was added to 10mℓ of 1mM silver nitrate solution and stirred with a magnetic stirrer for 24 hours. The mixture was then centrifuged at 12000 rpm for 10 minutes, and the supernatant was removed with a dropper. The pellet (silver nanoparticles) was resuspended in 9mℓ of deionised water. The process of centrifugation, decantation, and resuspension in water to remove the algae extract and unreacted silver nitrate was performed thrice, yielding the silver nanoparticle solution. The presence of the silver nanoparticle solution was confirmed by observing for a colour change from green (for *Ulva*) or brown (for *Sargassum*) to red.

Analysis of silver nanoparticles

The absorption spectra of the solutions were measured with a UV-vis spectrophotometer to confirm the presence of silver nanoparticles. Scanning electron microscopy was also conducted to analyse the size of the silver nanoparticles.

Preparation of bacterial cultures

Escherichia coli ATCC25922, Staphylococcus epidermidis ATCC12228, and Escherichia coli OP50 were inoculated separately in 10 mℓ of LB broth and incubated with shaking in an orbital shaker at 30°C for 24 hours. The absorbance of the bacterial cultures at 600 nm were standardised at 0.8.

Antibacterial test

0.5mℓ of bacterial culture, 4.5mℓ of LB broth, and 5mℓ of either silver nanoparticle solution for the test setup, or sterile water for the negative control setup, were mixed in a centrifuge tube and incubated with shaking in an orbital shaker at 30°C for 24 hours. Serial ten-fold dilutions

with 0.85% saline solution were then conducted to the appropriate dilution factor. 100μℓ of the diluted cultures were pipetted onto a LB agar plate, and spread evenly with a L-shaped cell spreader. The agar plates were then incubated in an incubator at 30°C for 24 hours. The number of colony forming units was then counted.

DPPH antioxidant test

1.9mℓ of methanol, 1mℓ of DPPH solution, and 0.1mℓ of either silver nanoparticle solution for the test setup, or sterile water for the negative control setup, were mixed together in a centrifuge tube. For the blank setups, 1mℓ of methanol was added in place of the DPPH solution. The setups were left in darkness for 20 minutes, and the absorbance readings were taken at 517 nm. The radical scavenging activity was calculated as follows:
[(final absorbance of control – final absorbance of test) ÷ final absorbance of control] x 100%

***Caenorhabditis elegans* N2 cytotoxicity test**

NGM agar was prepared by mixing 0.9 grams of sodium chloride, 7.5 grams of agar, 0.75 grams of bacto peptone and 300 ml of deionized water. The mixture was then autoclaved at 121 deg C and 15 psi for 15 minutes. After autoclaving, 0.3 ml of 5mg/ml cholesterol solution, 0.3 ml of 1M magnesium sulfate solution, and 7.5 ml 1M pH 6.0 potassium phosphate buffer was added to the mixture. 20 ml of the mixture was poured into a petri dish and cooled down to solidify the agar.

50μℓ of *E. coli* OP50 bacterial culture was added to a NGM agar plate. The NGM plate was then incubated for 24 hours at 30°C. A block of agar containing *C. elegans* N2 was then placed on the NGM plate. The NGM plate was then incubated at 20°C for another 48 hours. The *C. elegans* and *E.coli OP50* were then collected in M9 buffer and filtered through a 8 μm membrane filter. The residue (*C. elegans*), was resuspended in M9 buffer.

100 μℓ of either silver nanoparticle solution for the test setups, or sterile water for the negative control setups, was spread on the surface of a new NGM plate. 100μℓ of the *C. elegans* suspended in M9 buffer was then pipetted onto the center of a NGM plate. The plates were then incubated in an incubator for 24 hours at 20°C. The plates were then observed under a microscope, and the percentage survival of *C. elegans* was determined. *C. elegans* were considered alive if they moved.

Data Analysis

The Kruskal Wallis H Test was performed to determine if there was significant difference in the mean values of the setups. Differences were significant if the p value is less than 0.05.

3. Results and Discussion

3.1 UV-Vis Spectrophotometry

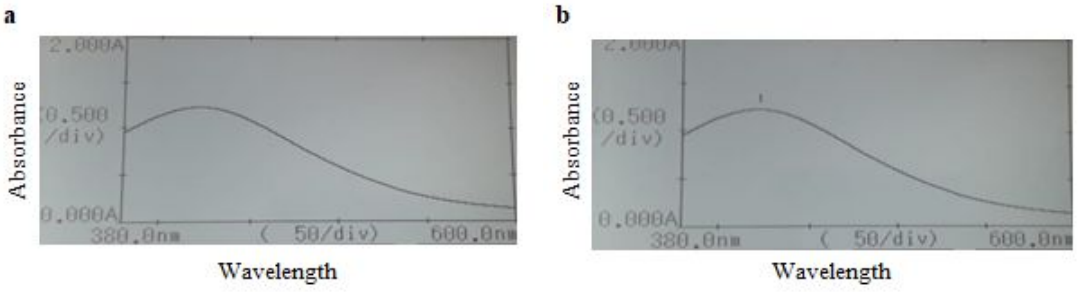

Figure 1. *Absorption spectra of Ulva (**a**) and Sargassum (**b**) synthesised nanoparticles. Wavelengths expressed in nanometers (nm).* (own image)

Ulva synthesised nanoparticles had a peak absorbance at 414.20 nm, while *Sargassum* synthesized nanoparticles had a peak absorbance at 422.20 nm. The wavelengths around 420 nm suggests that silver nanoparticles were synthesized (Zohreh, Morteza, Ahmad, & Arash, 2014), suggesting that *Ulva* and *Sargassum* extracts could synthesise silver nanoparticles.

3.2 Scanning Electron Microscopy

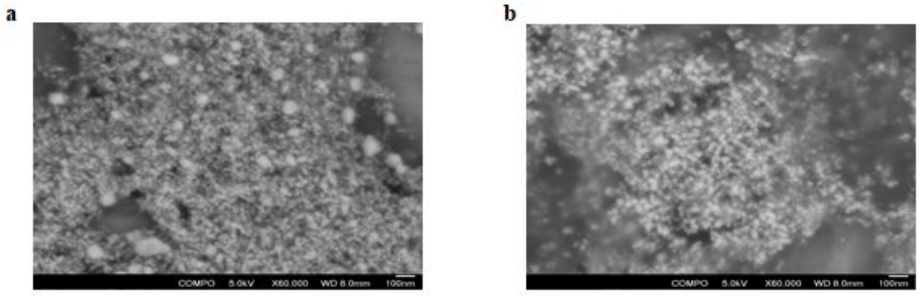

Figure 2. *Scanning electron micrographs of Ulva (**a**) and Sargassum (**b**) synthesised nanoparticles.* (own image)

As seen in Figure 2, there was a wide range of sizes for the silver nanoparticles, though the majority of the nanoparticles were between 30 and 50 nanometers in diameter.

3.3 DPPH Test

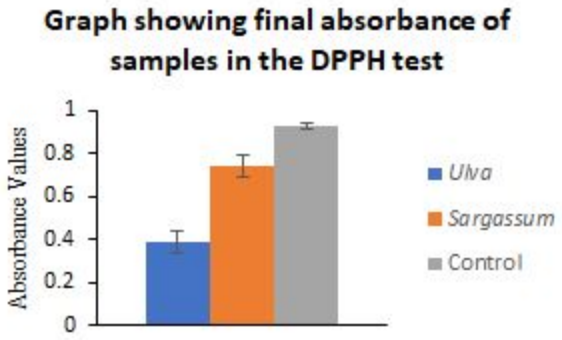

Figure 3. *Absorbance of solutions with respect to blanks 20 minutes after addition of silver nanoparticles. Sterile water was used as the control. Results presented as Mean ± Standard Error. n=5, p<0.01 when the Kruskal Wallis H-Test was performed.*

As seen in Figure 3, there was a significant ($p<0.05$) decrease in absorbance values when both *Ulva* and *Sargassum* synthesised nanoparticles were added. The free radical scavenging activity (FRSA) for *Ulva* synthesised nanoparticles was 58.47%, while that of *Sargassum* synthesised nanoparticles was 20.02%.

This shows that plant-synthesised silver nanoparticles do indeed display strong antioxidant properties, and may be used as a free radical scavenger, potentially mitigating the potential impact of excess free radical production when applied to human cells, making it suitable for *in vivo* applications. This finding is supported by other studies, which show similarly strong antioxidant properties of silver nanoparticles synthesised with other extracts. Mohanta et al. (2017) reported that the aqueous extract of *Erythrina suberosa* synthesised silver nanoparticles with a FRSA of 40% at concentrations of 20 $\mu g m\ell^{-1}$, which was higher than that of Butylated hydroxytoluene (BHT), at 8%, a commercial chemical antioxidant food additive. The antioxidant properties are likely due to the phytochemicals that are attached to the surface of the nanoparticles like, that are reducing agents (Ivanova et al., 2018)

3.4 Antibacterial Test (Colony Count Test)

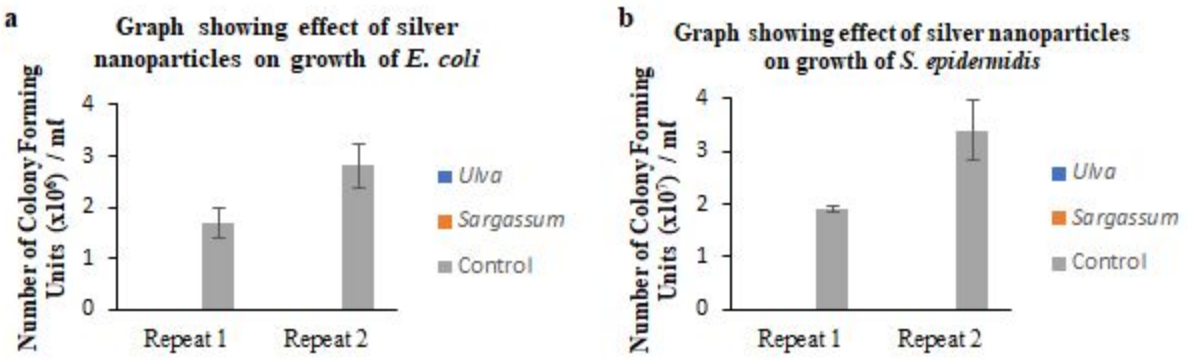

Figure 4. *Number of E. coli (**a**) and S. epidermidis (**b**) colony forming units per milliliter (mℓ) of incubated solution after addition of silver nanoparticles. Sterile water was used as the control. Results presented as Mean ± Standard Error. n=5, p<0.01 when the Kruskal Wallis H test was performed*

As seen in Figure 4, there were no *E. coli and S. epidermidis* colony forming units when *Ulva* and *Sargassum* synthesised nanoparticles were added. There were 1.69×10^6 and 2.81×10^6 colony forming units for the *E. coli* control, and 1.89×10^7 and 3.40×10^7 colony forming units for the *S. epidermidis* control, for the first, and second repeat respectively. The significant difference ($p<0.05$) between the tests and control setups suggests that silver nanoparticles were bactericidal against both *E. coli* and *S. epidermidis*.

The results suggest that the synthesised nanoparticles displayed strong antibacterial properties against both gram-positive and gram-negative bacteria, potentially making the synthesised nanoparticles suitable for use as a prophylaxis.

The finding was similar to those from Ivanova et al. (2018), which showed that silver nanoparticles synthesised with the extracts of aloe, geranium, and magnolia displayed strong bactericidal against both gram-negative (*Pseudomonas* and *Salmonella*) and gram-positive (*Bacillus* and *K. rhizophila*) bacteria.

3.5 *C. elegans* Cytotoxicity Test

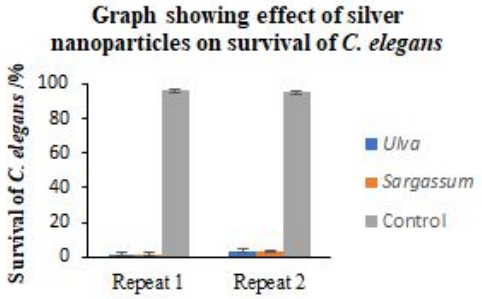

Figure 5. *Percentage survival of C. elegans after addition of silver nanoparticles. Sterile water was used as tje control. Results presented as Mean ± Standard Error. n=5, p<0.01 when the Kruskal Wallis H Test was performed.*

As seen in Figure 5. The survival percentage of *C. elegans* was 1.52% and 3.48% when Ulva synthesised nanoparticles were added, 1.51% and 3.26% when Sargassum synthesised nanoparticles were added, which were significantly ($p<0.05$) lower than the control, at 96.29% and 94.99%, for the first, and second repeats respectively. Thus, they synthesised nanoparticles were cytotoxic against *C. elegans*.

The results seemed to contradict other *in vivo* studies that showed little cytotoxic effects of silver nanoparticles. Ji et al. (2007) reported that there was neither significant change in body weight of mice, nor any significant change in the hematology and blood biochemical values after 28 days of exposure to silver nanoparticles via inhalation. This discrepancy may be due to the size of the nanoparticles. As can be seen in Figure 6, smaller nanoparticles tend to be more cytotoxic, while larger nanoparticles display little cytotoxic activity. Thus, the cytotoxicity exhibited may have been due to the presence of smaller silver nanoparticles.

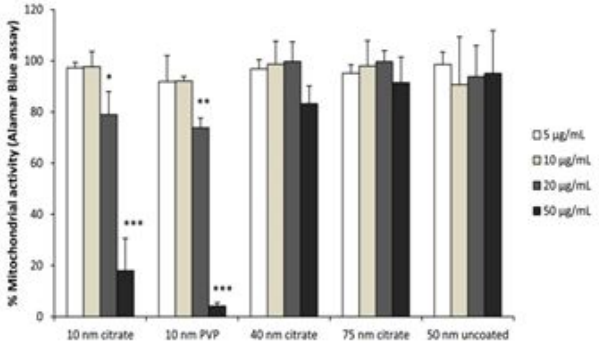

Figure 6. *Mitochondrial activity of human lung cells after exposure to different sizes and concentrations of silver nanoparticles* (Gliga et al., 2014)

4. Conclusion and Recommendations for future work

The extracts of *Ulva* and *Sargassum* were able to synthesise silver nanoparticles. The silver nanoparticles displayed strong antioxidant properties, and were bactericidal against *E. coli* and *S. epidermidis*. However, they also displayed cytotoxic properties, making them unsuitable for use as an antibacterial prophylaxis. Nevertheless, the nanoparticles synthesised still may have other applications, such as for use in killing cancer cells, or for use as antimicrobial coatings. Thus, this research has demonstrated the potential of using the extract of *Ulva* and *Sargassum* for synthesizing silver nanoparticles, which has economic and environmental implications, helping to make use of abundant, renewable, and affordable algal resources, as well as reducing the use of toxic chemicals with a high climate change potential.

In future research, changes could be made to the protocol to reduce cytotoxicity, such as using sucrose mediated centrifugation to remove the small nanoparticles (Suresh et al., 2015), and incorporating the nanoparticles into a cellulose hydrogel to improve the stability of the nanoparticles (Massmood et al., 2019).

Chang, Pan, Lin and Chang (2019) found *in vitro* that albumin conjugated silver-diamond nanohybrids were able to display strong, bactericidal properties against *E. coli* that lasted for more than 36 days, with low cytotoxicity towards human fibroblasts, lung adenocarcinoma epithelial cells, and breast adenocarcinoma cells, even at high concentrations of 500 $\mu g m\ell^{-1}$. However, the silver nanoparticles in Chang et al. (2019) were synthesized using the chemicals sodium citrate and sodium borohydride, which as discussed previously, are not environmentally friendly. Thus, further research can also look into the possible use of algae extracts in the synthesis of albumin conjugated silver-diamond nanohybrids, and the antibacterial, as well as cytotoxic effects of these nanohybrids for their effectiveness as a surgical prophylaxis.

Antifungal and antiviral properties of Ulva and Sargassum synthesised nanoparticles could also be investigated in future research, to see if they can be effective in preventing viral and fungal surgical site infections.

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