Original Article

Silver Nanoparticles Biosynthesized from *Vaccinium myrtillus* L. against Multiple Antibiotic Resistance and Biofilm Forming *Escherichia coli* and *Pseudomonas aeruginosa*

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ABSTRACT

Background: Despite new innovations and process improvements, biofilm forming bacterial infections still pose a serious threat to patients. Silver nanoparticles (AgNPs) have been shown to have antibacterial properties and have been applied for surface manufacturing of many permanent medical devices at the same time. Therefore, we attempted to compare the performance of green synthesis of AgNPs and *Vaccinium myrtillus* L. plant extracts in terms of antibacterial and antibiofilm potential against multi drug resistant (MDR) biofilm forming *Pseudomonas aeruginosa* and *Escherichia coli* clinical strains. **Materials and Methods:** The biosynthesized AgNPs were characterized by UV-Visible spectroscopy. The antibacterial activity of the nanoparticles was determined by using disc diffusion and broth micro dilution method. Antibiofilm properties of nanoparticles have also been investigated by using scanning electron microscopy (SEM) and tissue culture plate (TCP) method. **Results:** Both extract and AgNP showed comparable bactericidal ($p<0,0001$) and antibiofilm activity ($p<0,0001$), but the mode of bacterial interaction and the degree of damage were completely different. **Conclusion:** For the first time with this study, extracts and also nanoparticles obtained from *V. myrtillus* were found to be effective in strains that have high biofilm activity and multiple drug resistance. Biosynthesized AgNPs were found to reduce planktonic cells as well as biofilm growth in a dose dependent manner. The results also supported the antibiofilm potential of AgNPs. This finding thus provides an idea of the development of silver nanoparticle-based biomaterials for use as effective surface modifying agents.

Key words: *E. coli, P. aeruginosa,* Biofilm, *Vaccinium myritillus,* Nanoparticle.

INTRODUCTION

The human health problems have increased in spite of major advances in science and technology today. The infectious diseases is one of the most important of these problems. Micro-organisms gain resistance to existing antimicrobial drugs, especially antibiotics. Management of acute bacterial skin and skin structure infections with a focus on patients at high risk of treatment failure.[1] The most important reason for this is the wrong and unnecessary use of antibiotics. This is made comprehension of new drugs mandatory. Thus, the use of natural resources has come to the agenda again.[2] *Vaccinium myrtillus*, which has been used as food for centuries, has gained medical importance and has been the subject of scientific studies.[3] For infection to occur, the pathogenic micro-organism must first colonize the target surface. The colonization begins with the attachment of the micro-organism to living and inanimate surfaces. Micro-organisms attaching to foreign bodies from inanimate surfaces form

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biofilms. The role of biofilm in the pathogenesis of some chronic human infections is now widely accepted. The biofilm formation of *Escherichia coli* and *Pseudomonas aeruginosa* is responsible for serious infections in patients.[4-6] Increased microbial resistance to traditional antibiotics has been hampered, as well as increased biofilm formation in some micro-organisms, highlighting the development of many antimicrobial NP, including AgNPs. In this study, the antimicrobial and antibiofilm effect on the biofilm-forming clinical strains was tested using *V. myrtillus* extract and silver nanoparticles (AgNPs) synthesized by extract using green synthesis.

MATERIALS AND METHODS

Plant material and Preparation of anthocyanin rich extract

Vaccinium fruits was collected from province Balikesir of Turkey in May 2016. The method of anthocyanin-rich extract preparation was modified from Bagchi *et al.* (2004). The seeds of the fresh fruits were removed and cut into small pieces by passing through a shredder. The weighed 100 grams of fresh fruit was placed in a 500 mL beaker containing 100 ml of distilled water. The mixture was microwaved (900 W) for 2 minutes. At the end of the process,Vaccinium extract was filtered and stored at -20°C for further use.

Green Synthesis and Characterization of *Vaccinium myrtillus* L. Silver Nanoparticles

The extract is diluted to 50% and taken up in 2 mL of 50 mM HCl with 5 mL of $AgNO_3$ nanoparticle was synthesized using the method of Zheng *et al.* (2015).[8] Characterization of silver nanoparticles was carried out by UV-vis spectrophotometer.

Bacterial strains

Multi drug resistant strains of *Escherichia coli* and *Pseudomonas aeruginosa* were identified by the automated VITEK® 2 system (bioMérieux) and obtained from Microbiology Laboratory, Necip Fazil City Hospital, Kahramanmaras. Total of 50 clinically resistant strains, 10 of them to have high biofilm activity with methods of qualitatively SEM and quantitatively by tissue culture plate methods. All methods were repeated three times and these 20 strains were included in the study.

Antibacterial Activity of Extracts and Nanoparticles

Antibacterial activities of all twenty strains were studied according to Clinical and Laboratory Standards Institute: According to CLSI standards using by Kirby Bauer disk diffusion method[9] measuring of inhibition zones and micro dilution method[10] measuring of the minimum inhibitory concentration (MIC) values against extract and NPs.

Detection of Biofilm

Biofilm-forming strains were assessed by scanning electron microscopy (SEM, ZEISS LS-10 Life Science) and tissue culture plate methods. The isolated micro-organism was inoculated in 10ml of trypticase soy broth (with 1% dextrose) and incubated at 37°C for 24 hr. The incubated broth was diluted 1:100 with fresh trypticase soy broth (with 1% dextrose). 100µl of the broth culture was added to 10 ml of fresh trypticase soy broth (with 1% dextrose) using sterile micropipette tip and mixed well. Each of the three individual wells of sterile 96- well flat bottom polystyrene tissue culture treated plates was inoculated with 200µl of the diluted broth culture. For positive control, *Staphylococcus aureus* ATCC 25923 and *Pseudomonas aeruginosa* ATCC 27853 were used and they were incubated, diluted and added to tissue culture plates similar to the test isolate. For negative control, each of the three individual wells of the sterile 96-well flat bottom polystyrene tissue culture treated plates was inoculated with 200µl of the uninoculated sterile trypticase soy broth with 1% dextrose. The tissue culture plates were incubated at 37°C for 24 hr. After incubation, the contents of each well were removed by gentle tapping and the wells were washed four times with 200µl of phosphate buffer saline to remove free floating bacteria. After washing, 200µl of 2% sodium acetate was added to each well and incubated for 15 min to fix the biofilm. After fixing, the plate was emptied and air-dried. 200µl of 0.1% crystal violet was added to each well and incubated for 15 min to stain the fixed biofilm. The excess stain was removed by washing with deionized water and the plate was air-dried. The optical densities (OD) of wells were obtained using ELISA reader (Ao Absorbance Micro plate Reader-Azure Bio systems) at a wavelength of 570 nm. The mean OD value of negative control (ODnc) was calculated. The cutoff value was calculated from the ODnc, using the formula: ODco=ODnc+ 3x standard deviation (SD) of nc Average OD value of the test organism wells was calculated. Based on the OD value, the test organisms were classified according to Cristensen *et al.* (1985)[11] as in mean OD values <0.120=None, 0.120-0.240=None/weak and ≥0.240=High.

Data analyses

The extract and Np were analyzed three times and mean ± SD was calculated. Graphs and statistical analysis

were performed using GraphPad prism version 8.0.1 and Student *t*-test. *P* value less than 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Particle size and Characterization of *Vaccinum myrtillus* L. Silver Nanoparticles

The particle size of the synthesized AgNPs was determined by SEM and characterized by UV-vis spectrophotometer. The formation of silver nanoparticles (AgNP) was confirmed by ultraviolet visible spectroscopy, where a surface-plasmon resonance absorption peak was observed between 420 and 430 nm. The results are shown in Figure 1A and 1B.

Visualization of biofilm by SEM

The 400 mesh copper grids (Electronmicroscopy sciences, Hatfield, PA)were used for *in situ* visualization (Figure 2) of biofilm by SEM.

Antimicrobial and AntiBiofilm Activity

The extract and NPs were diluted at 1: 1, 1: 5, 1: 10, 1: 15, 1: 20, 1: 25,1: 30 ratio and antimicrobial activities were studied. Antimicrobial activity was not observed against *P. aeruginosa* strain 2,5,6,9 at concentration range. The antimicrobial activity of NPs was very effective than extract. Comparing the nanoparticle with the extract among the different MIC ratios, it is seen that the nanoparticles are effective at 1 to 2 fold less dilutions

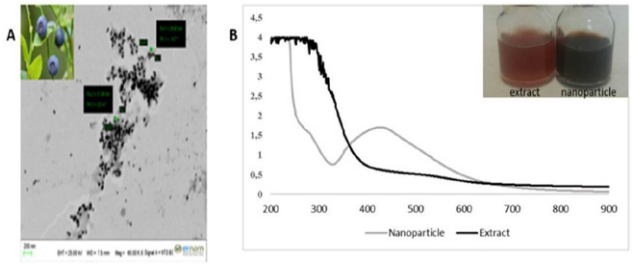

Figure 1: A) SEM image of nanoparticle and their size B) UV-vis spectrophotometer values.

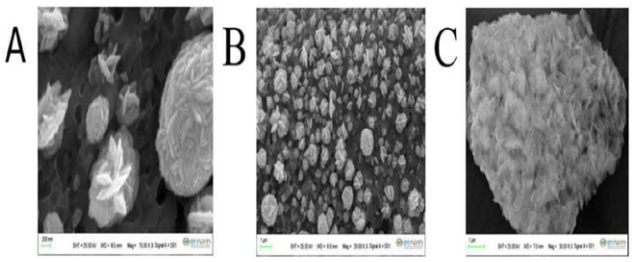

Figure 2: SEM image of biofilm formation A-B) 48 hr C)72 hr incubation result.

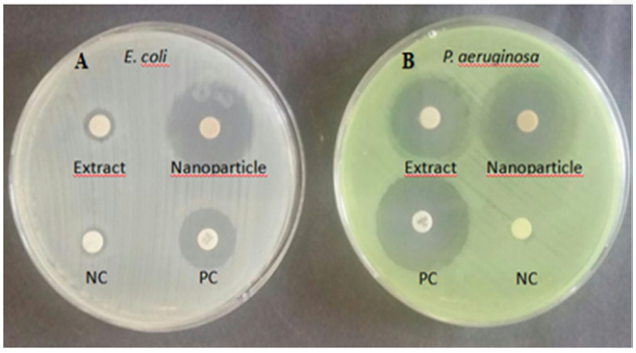

Figure 3: Disc diffusion results of A) *E. coli* B) *P. aeruginosa*.

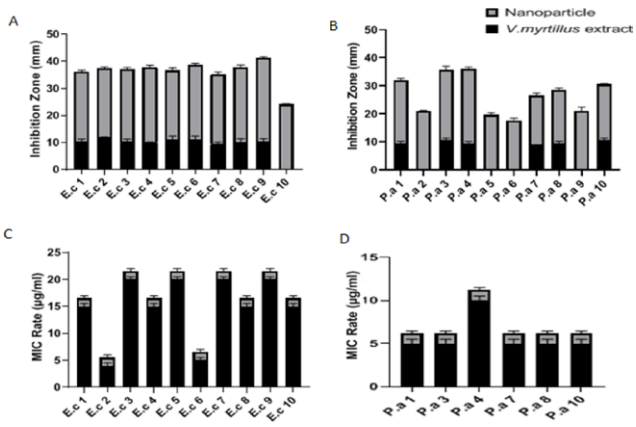

Figure 4: Antimicrobial activity results A-B) Disc Diffusion C-D) MICs.

*E.c=*E.coli*; P.a=*P.aeruginosa*

than the extract. All results were given Figure 3-4 and significant statistically (p<0,0001).

The changes in the biofilm at the end of exposure (2, 8, 18, 24hr) of the micro-organisms with extract and NPs were evaluated. The positive effect of samples on *E. coli* strains were observed at the end of 8 hr. But their effect on *P. aeruginosa* strains were effective after 18 hr by only dissolving in biofilm. For the detection of changes in biofilms, tissue microplate method was evaluated by at 2, 8, 18, 24 hr and compared with SEM images. As a result, the antibiofilm effect of the NP was about 15-fold higher than the extract for *E. coli* isolates and the biofilm formation was reduced higher than to non-to-weak range after 8 hr (p<0,0001). The antibiofilm effect for *P. aeruginosa* isolates was about 5 times higher observed in NPs and its effect decreased from high to medium degree after 18 hr (p<0,0001). The results of quantitative antibiofilm effect were similar to the qualitative SEM results as seen Figure 5.

As seen in Figure 3, it is seen that extracts and nano-particles are more effective on *E. coli* biofilm than *P. aeruginosa*. Especially nanoparticles disrupt *E. coli* cell

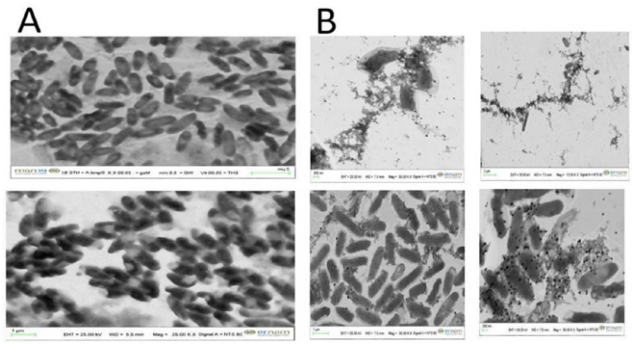

**Figure 5: SEM images of Biofilm formation (above -*E. coli*; below-*P. aeruginosa*).
A) Extracts B) NPs**

wall, while on *P. aeruginosa*, the extract partially decreases the biofilm layer, however nanoparticles enter the bacteria.

Although there are some antimicrobial studies done with *Vaccinium myrtillus*, there is no contradiction regarding antibiofilm feature. For the first time with this study, extracts and also nanoparticles obtained from *V. myrtillus* were found to be effective in strains that have high biofilm activity and multiple drug resistance.

Benzoic acid is a simple aromatic carboxylic acid and is found naturally in many plant species. Many *Vaccinium* species, such as *Vaccinium myrtillus*, contain considerable amounts of benzoic acid in the fruits.[12-16] The representative data obtained for benzoic acid derivatives such as vanillin, vanillic acid[17] and gallic acid,[18] has a biofilm inhibiting effect. This high antibiofilm effect may have been due to such compounds found intensely in this plant extract.

CONCLUSION

In this study, a simple and environmentally friendly method for AgNP synthesis is reported using *V. myritillus* extract. AgNPs were successfully synthesized from extract by green synthesis using silvernitrate solution. The effect of extract and AgNP on the biofilm-forming clinical strains have beentested. Theresults of this study suggest that biofilm production should be investigated clinically in multi-drug resistant strains and that treatment of micro-organisms may be more easily achieved if biofilm inhibition is provided. Extract incorporated AgNPs are promising effect on biofilm formations.

ACKNOWLEDGEMENT

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ABBREVIATIONS

AgNPs: Silver nanoparticles; **MDR:** Multi drug resistant; **SEM:** Scanning electron microscopy; **TCP:** tissue culture plate method; **MIC:** The minimum inhibitory concentration; **OD:** Optical density.

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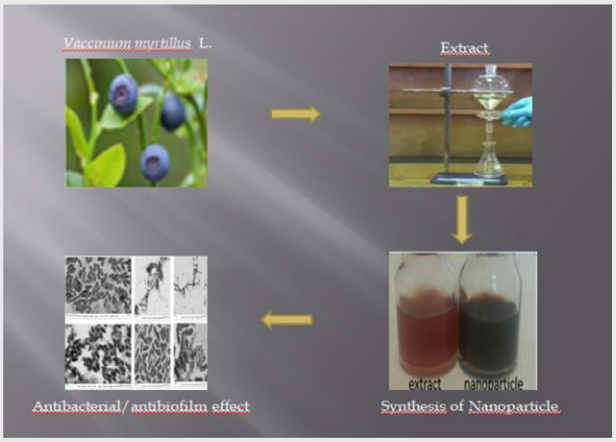
PICTORIAL ABSTRACT



SUMMARY

- The antimicrobial and antibiofilm effect on the biofilm-forming clinical strains was tested using *Vaccinium myritillus* L. extract and silver nanoparticles (AgNPs) synthesized by extract using green synthesis.
- The plant extracts and AgNPs that were synthesized from extract found to be effective on multidrug resistant Gram-negative bacteria.

About Authors

Dr. Nilay Ildiz, is the Head of Pharmaceutical Microbiology Dept in Erciyes University, Faculty of Pharmacy. She obtained her doctorate degree in Inönü University, Faculty of Medicine, Dept of Medical Microbiology in 2014. She continues to work on potential microbiological activities, Deveplopment of rapid disagnosis methods and recently on green chemistry.

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