Official Journal Issued by Faculty of Veterinary Medicine

Benha Veterinary Medical Journal

Journal homepage: https://bvmj.journals.ekb.eg/

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Original Paper

Antibacterial and antibiofilm activity of aqueous extract of nigella sativa silver nanoparticles against *Staphylococcus aureus* isolated from milk and dairy products

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ARTICLE INFO

Keywords

Staphylococcus aureus

Black cumin

Antibacterial, antibiofilm

Dairy products

Received 22/09/2024
Accepted 16/10/2024
Available On-Line 31/12/2024

ABSTRACT

Staphylococcus aureus (S. aureus) is a significant microorganism affecting both humans and animals. It has a reputation for being able to generate biofilms, which enhance its resistance to conventional antibiotics. The present study aimed to investigate the potential impact of silver nanoparticles based on the aqueous extract Nigella Sativa (Ns-AgNps) against *S. aureus* isolated from milk and some dairy products (yogurt and kareish cheese), then evaluate their biofilms formation. The dairy samples were collected from September (2022) to January (2023), with an overall amount of (100) samples, including (54) raw milk, (24) yogurt, and (22) kareish cheese, the samples were collected from local markets at El Quanater El Khairiya City, Egypt. The results showed a prevalence of *S. aureus* 29.6% in raw milk, 9.09% in kareish cheese, and 8.33% yogurt. the isolated *S. aureus* isolates were assessed using a microtiter plate test for the formation of biofilms and resulted in (45%) non-biofilm formers and (55%) weak biofilm formers. The outcomes demonstrated that the lowest concentration that inhibits bacterial growth (MIC) using Ns-AgNps ranged between (3.125-50 µg/mL) plating them showed no bacterial growth which indicated the same values for minimum bactericidal concentration (MBC). Also, the antibiofilm reduction rate on 20 *S. aureus* isolates using tissue culture plate assay ranged from 20.20 % to 93.20%. The results could open avenues for creating alternative antimicrobial and antibiofilm using natural extracts and nanoparticles, ultimately enhancing food safety and public health.

1. INTRODUCTION

Gram-positive *Staphylococcus aureus (S. aureus)* bacteria may grow in both oxygen-rich and oxygen-poor conditions, classifying it as a facultative anaerobe. It is considered a risk key pathogen for health hazards in human as well as veterinary medicine. It is known as a primary reason for mastitis in bovine (Dufour et al., 2012). Beyond animal health, *S. aureus* poses significant risks to public health due to its ability to contaminate animal-derived foods, including milk and dairy products (Hornik et al., 2021). This bacterium produces heat-stable enterotoxins (SE) that can lead to staphylococcal food poisoning, which is one of the most common foodborne illness causes in the world. (Kadariya et al., 2014; Fisher et al., 2018; Cvetnić et al., 2021). Besides foodborne diseases, *S. aureus* is accountable for several serious invasive illnesses, including nosocomial bacterial infections, surgical site infections, prosthetic joint infections, and respiratory tract infections (Cheung et al., 2021). *S. aureus* can cause diseases in both humans and animals along with its ability as a reservoir for infections that can be transmitted between animals and humans underscores its significant impact on both agriculture and public health (Haag et al., 2019).

Biofilms are complex microbial communities that provide bacteria with enhanced protection against antimicrobial and the immunological system of the host (Otto, 2008). Biofilm formation by *S. aureus* complicates the treatment, as these structures confer resistance to conventional antibiotics and facilitate persistent infections.

The need for alternate antimicrobial techniques is crucial given rising concerns regarding antibiotic resistance. Black cumin, also known as Nigella sativa (N. sativa), is a plant used in medicine. It is well known for having a wide range of pharmacological qualities, including antifungal, antibacterial, and anti-inflammatory effects (Gholamnezhad et al., 2016). The use of nanoparticle carriers has been proposed to enhance the efficacy of antimicrobial agents as well as drug delivery particularly against biofilm-forming bacteria (Zhou et al., 2018). Lately, researchers have explored the antibacterial potential of silver nanoparticles (AgNPs), that showed significant effectiveness against various pathogens (Rai et al., 2009). However, the synergistic effects of N. sativa extract-synthesized silver nanoparticles (Ns-AgNps) on *S. aureus* biofilms, particularly those isolated from dairy products, have not been thoroughly investigated.

Examining Ns-AgNps's inhibitory action against biofilm-forming *S. aureus* positive samples derived from milk and its derivatives in the Qalyubia Governorate was the aim of this current investigation. The primary objectives were to determine the prevalence of *S. aureus* in different examined dairy products and assess their biofilm-forming capabilities using a microtiter plate assay. Then understand the interactions between Ns-AgNps and *S. aureus* biofilms, this research sought for the creation of natural antimicrobial strategies for improving the safety of food and health of public.

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2. MATERIAL AND METHODS

The procedures followed in this study adhered to the animal care and use guidelines as sanctioned by the Ethical Approval Committee of the Faculty of Veterinary Medicine at Benha University, Egypt, under the ethical approval number. BUFVTM02-08-24

2.1. Samples collection

Between September (2023) and January (2024), a thorough collection of (100) raw milk and milk product samples was conducted. These samples consisted of (54) raw milk samples, (24) yogurt samples, and (22) kareish cheese samples. The collection procedure followed strict aseptic conditions and safety guidelines at the locations of purchase. Samples were randomly gathered from different local supermarkets, dairy stores, and villagers. in and around El Quanater El Khairiya city, Qalyubia Governorate, Egypt. Every sample was carefully gathered, labeled, and immediately stored in an icebox to maintain its integrity. They were then transported to the laboratory of the Animal Health Research Institute promptly to facilitate bacteriological examination.

2.2. Isolation and identification of staphylococci

This study employed buffered peptone water as a general medium and blood agar medium for enrichment. Baird-Parker agar (OXOID, ENGLAND) was utilized as a selective medium for *S. aureus*. For fifteen minutes, the media were sterilized at 121°C, following manufacturer specifications. Colonies displaying distinct staphylococcus characteristics were selected from the selective media and underwent Gram staining and biochemical tests, including Coagulase, Oxidase, and Catalase tests, following the protocol outlined by (Sneath et al.,1986).

As noted by (Quinn et al., 2002). Selected colonies were purified by repeated sub-culturing on selective media. The pure cultures were then transferred to a nutrient agar slant medium and incubated for 24 to 48 hours at 37°C. After that, the pure cultures were refrigerated at 4°C to ensure their preservation, and to maintain their viability for further analysis and experimentation.

2.3. Detection of biofilm using microtiter plate assay

In a sterile 96-well flat-bottom tissue culture plate, three wells were filled with a 200 µl bacterial slurry, while the positive control wells were left empty. For twenty-four hours, the plates were covered and incubated at 37°C. Following the incubation period, the wells' contents were removed using aspiration, and 250 µl of sterile physiological saline was used to wash each well three times. The non-adherent bacteria were eliminated by giving the plates a thorough shaking. After that, the wells were dyed for five minutes using 200 µl of 0.1% crystal violet. The plates were carefully rinsed with deionized water to get rid of any leftover stains, and then they were dried for 15 minutes at 40°C. To measure the production of biofilms, 200 µl of 95% methanol was subsequently added to each well. The plates were emptied and left to dry for fifteen minutes. Using an ELISA reader (model: Dawn R4, serial no: 610000079), the optical density (OD) of the stained adherent bacteria was measured at 620 nm, while the OD of the negative control was set to zero. (Stepanović et al., 2000). Each test's OD value was deducted from the mean OD value obtained from the media control well. The obtained corrected optical density values were employed as surface adhesion and bacterial biofilm development indicators. The samples were categorized as non-, weak, moderate, or strong biofilm producers.

2.4. Preparation of Sliver nanoparticle by aqueous black seed extract

Twenty milliliters (mL) of aqueous black seed extract and eighty milliliters (1 mM) of aqueous silver nitrate (AgNO3) solution -which was obtained from Sigma-Aldrich (St. Louis, MO, USA)—were combined in a round-bottom flask at a concentration of 0.02 mg/mL. Overnight, the mixture was continually mixed while it was at room temperature. The solution's color shifted from pale yellow to brown as the reaction went on, indicating the forming of nanoparticles. (Alkhathlan et al., 2020). This observation suggested the successful synthesis of silver nanoparticles, corroborating previous findings with slight modifications.

2.5. Antimicrobial activity of Ns-AgNps by MIC and MBC

We screened 20 isolates of *S. aureus*, some of which demonstrated biofilm production. The microdilution method, as described by Alekish et al. (2018), was employed to calculate the Ns-AgNPs' minimum inhibitory concentration (MIC).

Mueller-Hinton broth (MH broth) was used for the overnight incubation of bacterial cultures, and the turbidity was adjusted to meet a 0.5 McFarland standard using a nephelometer, resulting in an approximate bacterial concentration of $1x10^8$ CFU/ml (Andrews, 2001). MIC determination was performed using a 96-well microtiter plate. Initially, each well received 100 µl of MH broth culture added. Serial dilutions of the AgNs-NPs stock solution were prepared by adding 100 µl to the first well and using a 2-fold dilution method, resulting in concentrations ranging from 100 to 0.04µg/ml across the wells. The final concentrations of AgNs-NPs in each well were then adjusted by adding 100 µl of microbial culture to (50, 25, 12.5, 6.25, 3.125, 1.56, 0.78, 0.39, 0.195, 0.097,0.04,0.02 µg/ml). Following a 24-hour incubation period at 37°C, the well that showed no turbidity at all was determined to be the MIC. To verify bacterial growth, the inoculated broth was utilized as a negative control, whereas MH broth containing the predetermined bacterial concentrations was employed as a positive control. The experiment was conducted in three trials to calculate the average MIC.

Agar plates were streaked with 100 µL of the bacterial inoculum from the MIC well, and the plates were incubated overnight at 37°C to determine the minimum bactericidal concentration (MBC). (Almatroudi et al., 2020). The lowest concentration of Ns-AgNPs at which there was no sign of bacterial growth on the agar plate was known as the MBC.

2.6. Antibiofilm activity of Ns-AgNps

Using the tissue culture plate method, the anti-biofilm activity of biosynthesized Ns-AgNPs was assessed against biofilm-producing *S. aureus*. a widely accepted and standard assay for biofilm detection, as modified from (Balasamy et al., 2019). First, *S. aureus* was grown at 37°C for an entire night on blood agar plates. After that, one colony was put into each of the 100 mL conical flasks containing tryptic soy broth (TSB), and the cells were shaken at 100 rpm for six to seven hours, or until the concentration of bacteria in each flask was roughly 2.5×10^8 CFU/ml. After that, fresh TSB was added to the bacterial suspension at a ratio of 1:100 to get a concentration of about 10^6 CFU/mL. After being divided among 96-well flat-bottom microplates, this diluted suspension was incubated at 37°C for the whole night. Following incubation and washing, fresh TSB with different concentrations of Ns-AgNPs (50, 25, 12.5, 6.25, 3.125, 1.56,

0.78, 0.39, 0.195, 0.097 µg/ml) was added to each well's media without causing any disruptions to the biofilm. The plates were then subsequently incubated at 37°C 24h. After that, the medium was carefully taken out of each well, and sterile 1x phosphate-buffered saline (PBS) was used twice to wash out any non-adherent bacteria. The wells were then allowed to dry for 20 minutes at room temperature.

After 15 minutes of staining with 0.1% crystal violet solution, biofilms were gently washed with sterile PBS to get rid of any leftover stains. Thirty minutes were spent drying the plates. A microplate ELISA reader was used to detect the optical density (OD) at 595 nm after 100 µL of 95% ethanol was added to each well to quantify the biofilm. The following formula can be used to determine the percentage of biofilm inhibition:

Biofilm Inhibition (%) = ((OD of the untreated control - OD of the sample) ÷ OD of the untreated control) ×100.

A scale ranging from 0% to 100% was used to evaluate biofilm inhibition. Biofilm growth was promoted by negative values, biofilm inhibition was strongly indicated by values over 50%, and low anti-biofilm effectiveness was indicated by values between 0% and 50% (Adeyemo et al., 2022).

3. RESULTS

3.1. Isolation and identification of staphylococci

Staphylococci colonies, typically ranging from 1-3 mm in diameter, exhibited characteristic traits such as being circular, smooth, raised, and opaque, with a creamy consistency. Notably, most of *S. aureus*-positive samples showed β-hemolytic properties. Among the samples tested, raw milk exhibited the highest prevalence of *S. aureus*, with 29.6% (16/54). In contrast, both Kareish cheese and yogurt demonstrated lower prevalence rates for *S. aureus*, with only 9.09% (2/22) and 8.33% (2/24) (Table 1).

3.2. Evaluation of the capacity of S. aureus isolates to produce biofilms

No surface adhesion when OD ≤ ODc, weak surface adhesion when ODc < OD ≤ (2×ODc), moderate surface adhesion when (2×ODc) < OD ≤ (4×ODc) and strong surface adhesion when (2×ODc) < OD ≤ (4×ODc). Among 20 examined *S. aureus* positive samples, 9 samples (45%) did not exhibit any biofilm formation. In contrast, 11 samples (55%) displayed weak biofilm formation. Notably, no samples were observed to exhibit moderate or strong biofilm formation characteristics (Table 2).

3.3. Assessment of antimicrobial activity of Ns-AgNps on S. aureus by MIC and MBC

Determination of Ns-AgNps MIC against *S. aureus* isolates (n=20) in repeated three trials resulted in slightly varying MICs values. Approximately MIC values were 12.5, 25, or 50 µg/ml. The average of the three trials for each isolate gave a MIC value between 10.4166 and 41.6666 µg/ml except for one isolate that showed a lower MIC value (5.208 µg/ml). Shortly, the highest obtained Ns-AgNps MIC values against tested *S. aureus* isolates was 50µg/ml (Table 3). Plating of MICs value showed no bacterial growth on the agar plate, which indicated a significant bactericidal impact of Ns-AgNps on all tested *S. aureus* isolates as MIC and MBC values were the same.

3.4. Assessment of antibiofilm activity of Ns-AgNps

Evaluation of the antibiofilm reduction rate of Ns-AgNps against 11 weak biofilm former *S. aureus* isolates was done using the tissue culture plate method. It demonstrated that Ns-AgNps at different concentrations, ranging from 50-6.25 µg/ml, effectively reduced biofilm formation with reduction rates between 20.20% and 93%. The strong antibiofilm activity (≥50% reduction) was exerted by 50 µg/ml against 9/11 isolates, 25 and 12 µg/ml against 8 isolates, and 6.25 µg/ml against 4 of them. Otherwise, these concentrations exerted low anti-biofilm effectiveness (0%-50% reduction) against the rest of the isolates (Fig. 1). Concentrations lower than 6.25 µg/ml showed enhancement of biofilm growth as the calculated percentage of biofilm inhibition was less than zero.

Table 1 *S. aureus* Prevalence in Examined Dairy Samples

samples	No. of examined samples	+ve *S. aureus*	
		No	%
Raw milk	54	16	29.6
Karesh cheese	22	2	9.09
Yogurt	24	2	8.33
Total	100	20	20%

Table 2 Percentage of biofilm former *S. aureus* in examined samples

Biofilm formation	Number of samples	Percentage%
Weak	11	55
Moderate	0	0
Strong	0	0
No biofilm	9	45
Total	20	100

Table 3 MIC and MBC of Ns-AgNps on *S. aureus* isolated from Dairy Samples.

Samples	MIC 1	MIC 2	MIC 3	average
1	25	12.5	6.25	14.5833
2	25	12.5	12.5	16.6666
3	12.5	12.5	12.5	12.5
4	50	25	25	33.3333
5	6.25	3.125	6.25	5.20833
6	25	50	50	41.6666
7	25	25	12.5	20.8333
8	25	12.5	50	29.1666
9	12.5	12.5	12.5	12.5
10	12.5	25	50	29.1666
11	12.5	25	50	29.1666
12	50	25	50	41.6666
13	50	50	25	41.6666
14	25	25	12.5	20.8333
15	50	50	25	41.666
16	50	12.5	25	29.1666
17	25	50	25	33.3333
18	25	25	12.5	20.8333
19	12.5	12.5	12.5	12.5
20	12.5	6.25	12.5	10.4166

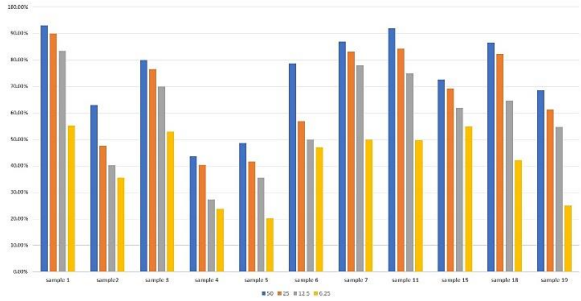

Fig. (1). Antibiofilm inhibition rate of Ns-AgNps against weak biofilm former *S. aureus*

4. DISCUSSION

The present study presented the variation in the prevalence of *S. aureus* across different dairy products. Raw milk exhibited the highest prevalence rate with 29.6% of the positive *S. aureus* samples. This finding suggested that raw milk may be a more frequent source of *S. aureus* contamination compared to processed dairy products like Kareish cheese and yogurt which showed *S. aureus* prevalence of 9.09% and 8.33%, respectively. In contrast to the current results, Zeinhom and Abed, (2020) found that the highest incidence of *S. aureus* was found in Kareish cheese (18%, 36/200), followed by low-salt (talaga) cheese (13%, 26/200) and raw milk (13%, 26/200). Also, Meshref et al. (2019) found the occurrence of *S. aureus* in kareish cheese (17%, 34/200) followed by small-scale yogurt (11%,

22/200) then (6.5%,13/200) in raw cow's milk and the lowest prevalence was in large scale yogurt (4.5%, 9/200) when examined samples collected from shops of dairy products and street peddlers in Beni-seuf governorate, Egypt. While *Methicillin-resistant Staphylococcus aureus (MRSA)* was found to be prevalent in raw milk and dairy products in 53% (106/200) of all milk and dairy products in Mansoura City, Egypt. The prevalence rates of MRSA were found to be 75% in raw milk, 65% in Damietta cheese, 40% in Kareish cheese, 50% in ice cream, and 35% in yogurt samples (Al Ashmawy et al., 2016).).

The data highlight the importance of rigorous hygiene and monitoring practices in dairy production, particularly for raw milk, which seems to be more prone to contamination by *S. aureus.* The results underscore the need for continued vigilance in guaranteeing the safety and quality of raw milk to prevent potential health risks associated with *S. aureus.*

A crucial element of *S. aureus's* pathogenicity is the formation of biofilms, which adds to the bacterium's ability to persist in various environments and resist treatment. The analysis of biofilm formation among the 20 *S. aureus* isolates revealed that a significant majority (55%) exhibited only weak biofilm formation, while a notable 45% did not form biofilm at all. Importantly, none of the isolates demonstrated moderate or strong biofilm-forming abilities. Interestingly, it has been recorded that the predominance of weak biofilm formation by *S. aureus* may have implications for its potential to cause chronic infections or resist antimicrobial treatment (Peng et al., 2022). In northern Xinjiang, China, Kou, and his co-operators gathered samples of raw milk from several animals across four distinct regions and assessed the ability of forming biofilm from positive *S. aureus* samples. They found 25 isolates out of 62 (40.3%) had a strong ability to form biofilm while 12/62 (19.4%) had a moderate ability to form biofilm (Kou et al., 2021).

The assessment of the antimicrobial activity of Ns-AgNps on *S. aureus* isolates revealed the bacteriostatic and bactericidal effect of Ns-AgNps against them. The concentrations spanning from (3.125 to 50 µg /ml) of Ns-AgNps effectively inhibited and prevented *S. aureus* biofilm growth. so, 50 µg /ml can be recommended as MIC and MBC of Ns-AgNps against *S. aureus*. The average of Ns-AgNps MIC values against the 20 *S. aureus* isolates was 24.84 µg/ml. These findings align with previous studies suggesting the ability of silver nanoparticles to fight against different bacterial strains, including *S. aureus* (Singh et al., 2008; Lara et al., 2010). The findings of Swalans and her team, who found that silver nanoparticles showed variable antibacterial efficacy against *staphylococci*, were also supported by the results of this investigation. The MIC ranged from 0.19 to 4000 µg/mL for *S. aureus* and from 1.14 to 4000 µg/mL for *S. epidermidis*. (Swolana and Wojtyczka, 2022)

Fortunately, it has been shown that N. sativa seed extracts can inhibit bacterial pathogens from forming biofilms by disrupting forces like Brownian motion, sedimentation, as well as electrostatic forces, which make it easier for bacteria to adhere to surfaces. (Roy et al., 2018). Certain inorganic and organic compounds, along with nutrients, are crucial for bacterial cell growth and adhesion (Sandasi et al., 2010). Plant extracts may limit the availability of these nutrients, while active compounds in the extracts can potentially reduce surface colonization and epithelial infections. (Chmagh et al. 2023). Also, Almatroudi et al. (2020) explained that Ns-AgNps had bacteriostatic, bactericidal, and antibiofilm activity, they discovered that low concentrations of Ns-AgNps reduced the growth of E. coli (15 and 30 µg/ml), K. pneumonia (15 and 30 µg/ml), P. aeruginosa (30 and 60 µg/ml), and S. aureus (6.5 and 15 µg/mL). Furthermore, Chmagh and his team employed Ns-AgNps as antibacterial and determined MIC on several types of bacteria such as K. pneumoniae (1.4 mg/ml), E. coli (1.1 mg/ml), and S. aureus (0.5 mg/ml) (Chmagh et al., 2023).

Moreover, the observed inhibitory effect on biofilm formation corroborates silver nanoparticles' promise as a cutting-edge strategy to fight bacterial biofilms, which are known for their increased resistance to conventional antimicrobial agents (Gurunathan et al., 2014). Here, using the tissue culture plate approach, Ns-AgNps' antibiofilm activity was evaluated. The results showed that Ns-AgNps dramatically reduced *S. aureus* biofilm formation, with inhibitory rates varying from 20.20% to 93.20% across the 11 weak biofilm former *S. aureus* isolates. Among the observations of Almatroudi et al. (2020), the Ns-AgNps antibiofilm effect against *S. aureus* was 82.84% reduction at a concentration of 12.5µg/ml through using the tissue culture plate method. Additionally, Chmagh et al. (2023) assessed biofilm formation inhibition using the crystal violet (CV) staining method. The findings showed that the application of N. sativa seed extract reduced the formation of biofilms in *K. pneumoniae*, *E. Coli*, *B. cereus,* and *S. aureus* in a concentration-dependent manner. The extract decreased the production of biofilms in these bacteria by8.92-65.91%, 7.03-71.55%, 9.46-67.5% and10.06-76.76% respectively, at increasing concentrations ranging from MIC/16 to MIC/2.

5. CONCLUSIONS

This study highlights the significant differences in *S. aureus* prevalence across various dairy products, with raw milk exhibiting the highest contamination rate at 29.6%, compared to lower rates of 9.09% in Karesh cheese and 8.33% in yogurt. Only 55% of *S. aureus* isolates displayed weak biofilm formation. Ns-AgNps demonstrated substantial antimicrobial activity, suppressing *S. aureus* efficiently at 50µg/mL to 3.125µg/ml concentrations and showed significant antibiofilm activity with inhibition rates from 20.20% to 93.20%. These findings emphasize the need for enhanced hygiene and monitoring in dairy production to reduce *S. aureus* contamination. It is suggested that Ns-AgNps could be a promising antimicrobial and antibiofilm agent for managing *S. aureus* infections.

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