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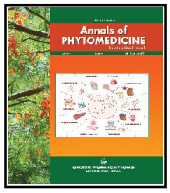

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Phytocompounds against uropathogens: Antibacterial and antifungal activities of *Nelumbo nucifera* Gaertn. methanolic extract against selected biofilm-forming pathogens

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Abstract

Uropathogens including bacteria and fungi are responsible for several diseases including nosocomial infections like catheter-associated urinary tract infections (CAUTI). When CAUTIs are caused by biofilms on catheter surfaces by multidrug-resistant microorganisms, it creates mild to severe complications due to their complex structure making the treatment and management challenging. Even though, there are many chemical catheter coating agents available, the researchers are looking for potential phytocompounds to be developed as coating agents as the micro-organisms find it difficult to develop resistance against them due to many reasons including the modes of action like targeting multiple sites in microbial cells. Thus, the present investigation analyses the antibiofilm and antimicrobial potentials of a plant, *Nelumbo nucifera* Gaertn. against CAUTI-causing organisms like *Enterococcus faecalis*, *Escherichia coli*, *Candida albicans*, and *Staphylococcus aureus*. Antimicrobial activities of the methanolic extract of the herb were tested by agar diffusion method and the minimal inhibitory concentration of the extract was found to be 0.5 mg/ml for *S. aureus*, 1 mg/ml each for *E. faecalis*, *E. coli*, and 2 mg/ml for *C. albicans*. The antibiofilm potential of *N. nucifera* was studied quantitatively by biofilm inhibition assay, and the extract successfully eliminated the mature biofilms of *S. aureus*, *E. faecalis*, *C. albicans*, and *E. coli*, and by 83%, 87%, 89%, and 83%, respectively. The activity of the extract as a catheter coating agent against all the test pathogens was also proved positively using an *in vitro* bladder model. The plant extract also showed promising antioxidant properties and when performing MTT assay using L_{929} cells. It was proven that the extract possesses no cytotoxic effect. So, the authors recommend further detailed studies to explore the chances of using *N. nucifera* based phytocompounds as a better alternative in the treatment of CAUTI and to be used as a catheter coating agent to prevent biofilm formation.

1. Introduction

Medical devices are used to improve the health of inpatients admitted for various reasons, and the continued usage of in dwelling devicesis often associated with microbial infections, resulting in unpredicted difficulties (Pietrocola *et al*., 2022; Yadav *et al*., 2020). The presence of medical devices in the human body provides the optimum microenvironment for the microbes to grow within the human body, resulting in medical device-related infections (Skelton-Dudley *et al*., 2019). An indwelling catheter is one of the most important medical devices used for cleaning the urethra and bladder by eliminating liquid waste (Milo *et al*., 2019; Wooller *et al*., 2018; Saint *et al*., 2016; Guggenbichler *et al*., 2011). The usage of catheters for longer periods may create CAUTIs, which are the third most important nosocomial infection among others that affect millions of lives globally (Yisiak *et al.,* 2021; Flores-Mireles *et al*., 2015).

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The presence of uropathogens in the catheter surfaces initiates the CAUTIs by entering the microbes from the external environment to inside the bladder and urethra and making microbial attachment, leading to colonization in the disinfected urinary system and causing low to severe complications resulting in high economic loss due to longer stay and increased mortality rate (Magill *et al*., 2018). Mostly, CAUTI comprises polymicrobial structures such as bacteria and fungi, making infection more challenging. Microorganisms like *E. faecalis, E. coli, S. aureus,* and *C. albicans* are frequently isolated microbes in CAUTI (Kurmoo *et al*., 2020; Di Martino, 2018). In a short period, these organisms form slimy three-dimensional structures embedded with extra polymeric substances that are a combination of proteins, DNA and polysaccharides,and they can form 500 µm thickness biofilms and support different bacterial cultures (Nadell *et al*., 2015; Rugaie *et al*., 2022) by protecting the organisms from various external sources like antibiotic treatment *via* several resistance mechanisms including the expulsion of antibiotics, target site alteration, *etc*. The biofilm-forming microbes entered into an inactive metabolic state to fight antibiotics and respond differently to chemotherapeutic agents (Bahamondez-Canas *et al*., 2018; Fulaz *et al*., 2020), thereby reaching bacterial tolerance, leading to the development of drug-resistant strains (Walker *et al*., 2020; Percival *et al*., 2015). The resistant bacteria have the ability to block the catheter and form polymicrobial infections, which worsen the

patient's condition. The changed microenvironment inside developed biofilms that contain accumulated mineral acids and their waste products play an important role in reducing antibiotic treatment and management of CAUTI, often harder due to the biofilm-forming ability of these organisms (Maharjan *et al*., 2018; Peng *et al*., 2018; Tenke *et al*., 2017). Even though, the chemical antimicrobial and antibiofilm agents are widely used, they are prone to the development of antimicrobial resistance and the scientific community is now searching for novel plant-based bioactive compounds that have less tendency to develop antimicrobial resistance developed due to many reasons including their modes of action like targeting multiple sites in and on microbial cells (Mishra *et al*., 2024; Ashraf *et al*., 2023; Jadimurthy *et al*., 2023; Khameneh *et al*., 2021).

For several decades, plants have been playing a vital role in drug discovery and also, many of the drugs in current use in the medical fields (Jyotsana, 2024; Ayman *et al.,* 2023; Shamna *et al*., 2022; Nasim *et al*., 2022; Veeresham, 2012). Plants havea wide range of biomolecules with potent pharmaceutical applicationsdue to their anti-inflammatory, antioxidant, antimicrobial, and antidiabetic properties (Singh *et al*., 2024; Parham *et al*., 2020). So, the present study selected *N. nucifera*, a monogeneric plant commonly known as the sacred Indian lotus from the Nelumbonaceae family that grows in Asian countries. Different parts of this plant have been used as herbal medicines to treat many diseases such as depression, heart problems, cancer, hypertension, diarrhea, and insomnia (Chen *et al.,* 2019; Sharma *et al*., 2017; Mukherjee *et al.,* 2009), and also as antiemetic, anthelmintic, and in the treatment of rashes (Arvind *et al*., 2023). Thus, the study investigates the antimicrobial and antibiofilm activities of the methanolic extract of pink *N. nucifera* flower against selected uropathogens like *E. coli, S. aureus, E. faecalis,* and *C. albicans.*

2. Materials and Methods

2.1 Plant authentication

The plant used in the present investigation was identified as *Nelumbo nucifera* Gaertn. by Dr. Shamna, Department of Herbal Medicine, Deseeya Ayurvedic Pharmacy, Calicut, India, with authentication number DAP/22-22/2024.

2.2 Inoculum preparation

The overnight cultures of selected uropathogens (*S. aureus, E. faecalis, E. coli,* and *C. albicans*) were adjusted to 0.5 MacFarland unit and were used for all the experiments. The cultures of *E. faecalis* and *S. aureus* were grown inbrain heart infusion (BHI) broth, *E. coli*on mueller hinton broth (MHB), and *C. albicans* was grown on sabouraud dextrose broth (SDB). The positive controls used were ampicillin, rifampicin, andnystatin, and the vehicle control was the solvent methanol.

2.3 *N. nucifera* methanolic crude extract preparation

N. nucifera flower powder purchased from the local market was weighed (20 g) and added to cellulose thimble which was placed in a Soxhlet apparatus as per the protocol (Harley *et al.,* 2022). The reaction was started when the methanol was added and it was continued for many hours till the clear solution attained. Finally, the solvent-evaporated and the crude extract was used in further studies.

2.4 Antimicrobial activity of *N. nucifera* crude methanolic extract

The antimicrobial potential of *N. nucifera* methanolic crude extract was determined against the test pathogens such as *E. coli, C. albicans, S. aureus,* and *E. faecalis* by well-diffusion standardized protocol (Meiyazhagan *et al*., 2016). The overnight cultures were swabbed onto the petri plates, and the plates were drilled and loaded with two different concentrations (1mg and 2 mg/well) of *N. nucifera* extract and allowed for 24 h to form zone inhibition around the drilled well, which indicated antimicrobial activity against test micro-organisms.

2.5 Determination of minimum inhibitory concentration (MIC) of *N. nucifera* methanolic crude extract

Using the standardized microdilution protocol (Meiyazhagan *et al*., 2015), the MIC of *N. nucifera* methanolic crude extract was calculated against the selected uropathogens. In a 96-well plate, the varying concentrations of *N. nucifera* crude extract were prepared by serially diluting 4 mg/ml of *N. nucifera* crude extract in respective broth until it reached 0.031 mg/ml, followed by cultures addition and allowed for 24 h to form turbidity. Later, the optical density of each of the wells was found at 600nm.

2.6 Effect of *N. nucifera* crude methanolic extract on biofilm formation

The effect of *N. nucifera* crude methanolic extract on the formation of biofilms by the selected pathogens was investigated using the crystal violet method (Meiyazhagan *et al*., 2015). The overnight test cultures were allowed to form biofilms in the presence of a wide range of *N. nucifera* methanolic crude extract concentrations from 4 mg to 0.031 mg/ml for 5 days in starvation conditions. Later, the attached biofilms were permitted for methanol fixing, followed by crystal violet staining for the subsequent addition of ethanol acetone mixture for destining, and the purple-coloured final product was analyzed at 570 nm.

2.7 Effect of *N. nucifera* crude methanolic extract on mature biofilm eradication

The effect of *N. nucifera* crude methanolic extract effect on mature biofilms formed by the test organisms was studied by using the crystal violet method (Meiyazhagan *et al*., 2015). In brief, 5-day maturedbio films of all the test pathogens were treated with 1X, 2X, and 3XMIC concentrations of *N. nucifera* methanolic crude extract followed by methanol fixation for attached biofilms. The crystal violet staining was performed and an ethanol-acetone solution was added to remove the stain on the biofilms. The end product was analyzed at 570 nm.

2.8 Antimicrobial activities of *N. nucifera* methanolic crude extract coated catheters

The catheter coated with *N. nucifera* methanolic crude extract was evaluated for its antimicrobial potential against *E. coli, C. albicans, S. aureus,* and *E. faecalis* using an *in vitro* bladder model (Goda *et al.,* 2022). A small piece of silicone catheter tube coated with *N. nucifera* methanolic crude extract was placed over the lawned test pathogen cultures on respective petri plates and incubated for zone formation around the tube, which indicated the antimicrobial activity of the *N. nucifera* methanolic crude extract against the test pathogens.

2.9 Antioxidant property of *N. nucifera* methanolic crude extract

The antioxidant property of *N. nucifera* methanolic crude extract was evaluated through a2, 2-diphenyl-1-picryhydrazyl (DPPH) free radical scavenging assay (Gayathri and Kumar, 2016). Briefly, *N. nucifera* methanolic crude extractin different concentrations (5 mg/ml, 4 mg/ml, 3 mg/ml, 2 mg/ml, 1 mg/ml, and 0.5 mg/ml) was reacted to DPPH solution for 30 min. The resultant was analyzed at 517 nm to calculatethe radical scavenging activity percentage of *N. nucifera*.

2.10 Cytotoxicity assay for *N. nucifera* methanolic crude extract

A cytotoxicity investigation was performed for *N. nucifera* methanolic crude extract on L_{929} (mouse fibroblast cells) through an MTT assay (Meiyazhagan *et al*., 2015). Shortly, the cells cultured in dulbecco's modified eagles medium (DMEM) were received concentrations (4 mg/ml, 3 mg/ml, 2 mg/ml, 1 mg/ml, and 0.5 mg/ml) of the extract and allowed to react for 24 h and permitted for formazan product by adding MTT solution. The percentage of the viability of the cell was analyzed after measuring the final product at 570 nm.

2.11 Statistical analysis

The mean and standard deviations were used for calculating error bars for all the experiments.

3. Results

3.1 Antimicrobial activity of *N. nucifera* methanolic crude extract

The antimicrobial activity of *N. nucifera* methanolic crude extract examined against the selected microorganisms is shown in Figure 1. As observed, the zone formation around the two different concentrations loaded well was observed, and the zone diameter was measured, which indicatedthe dose-dependent activity of *N. nucifera*.

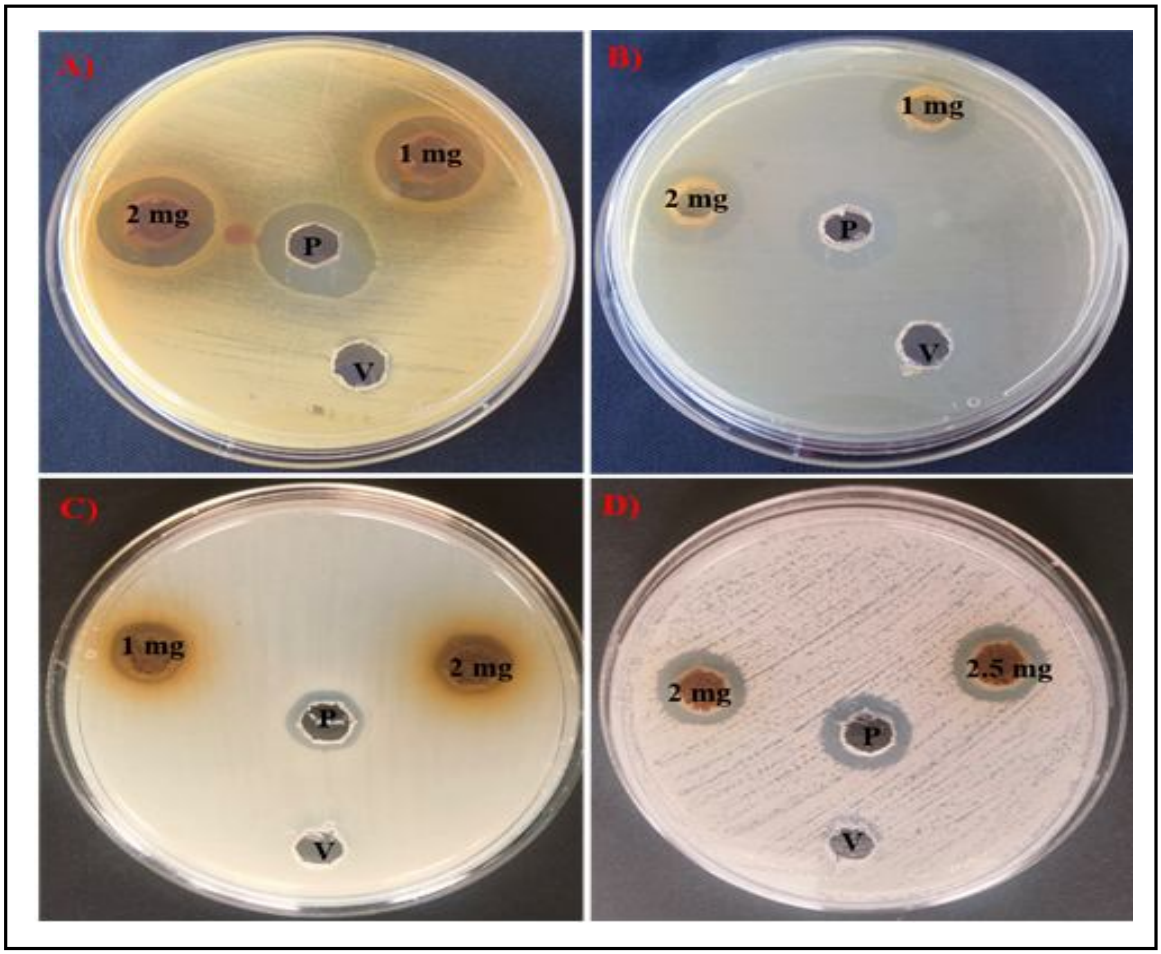


Figure 1: Antimicrobial activity of *N. nucifera* methanolic crude extract. (A) *S. aureus*, (B) *E. faecalis*, (C) *E. coli*, and (D) *C. albicans*. Note: P-positive controls and V-vehicle control.

3.2 MIC determination for *N. nucifera* methanolic crude extract

The MIC of *N. nucifera* methanolic crude extract determined against *E. coli, E. faecalis, S. aureus,* and *C. albicans,* is presented in Figure 2. The minimal growth inhibitory concentrations of *N. nucifera* methanolic crude extract were plotted and indicated that 0.5 mg/ml of *N. nucifera* methanolic crude extract was required to inhibit the growth of *S. aureus*. Similarly, 1 mg/ml of *N. nucifera* methanolic crude extract was needed for *E. faecalis* and *E. coli* to stop their growth, whereas 2 mg/ml of extract was required for *C. albicans* inhibition.

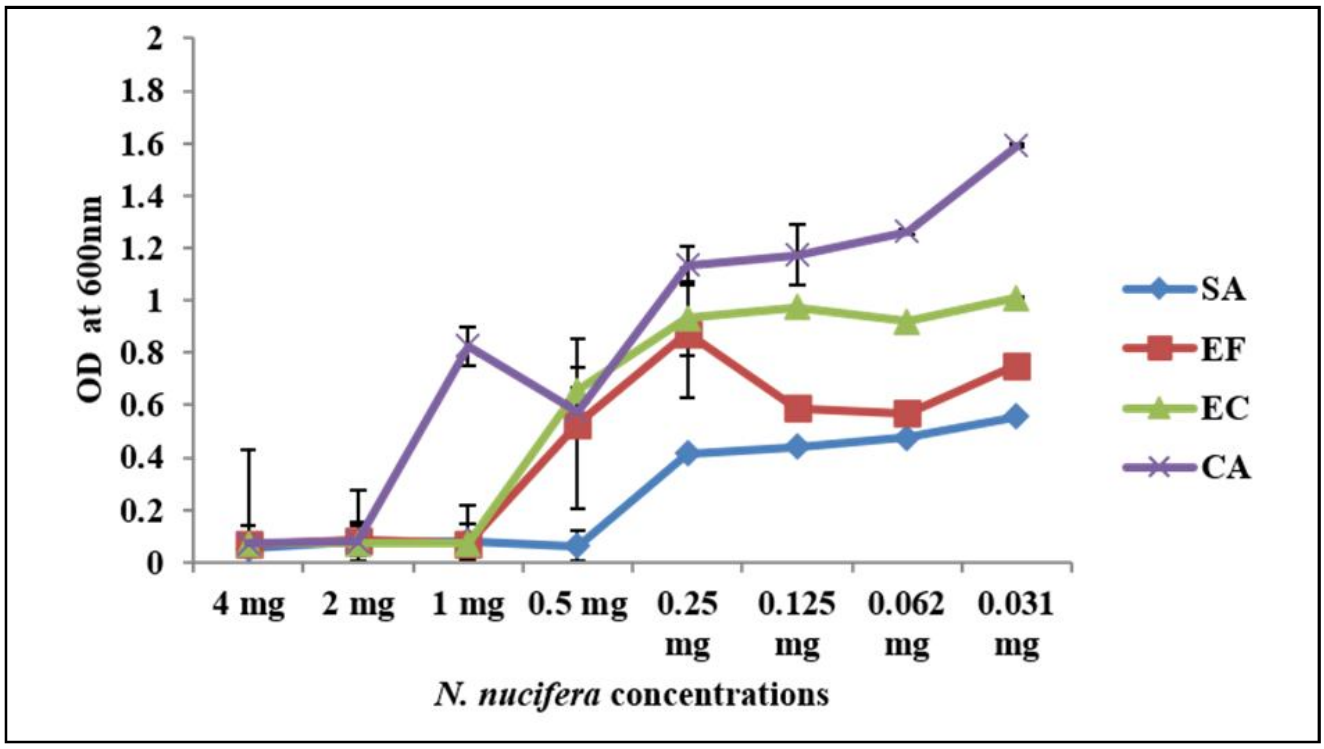


Figure 2: MIC determination of *N. nucifera* methanolic crude extract against *E. coli, S. aureus, C. albicans,* and *E. faecalis* using microdilution method. Note: SA-*S. aureus,* EF- *E. faecalis,* EC- *E. coli* and CA-*C. albicans*.

3.3 Effect of *N. nucifera* crude methanolic extract on biofilm formation

The effect of *N. nucifera* crude methanolic extract on the biofilm-forming capability of *S. aureus, E. coli, C. albicans,* and *E. faecalis*on non-living surfaces quantified are presented in Figure 3. The formation of biofilms after treatment with various concentrations of *N. nucifera* methanolic crude extract was quantified using crystal violet, and the percentage of biofilm formation was noted after MIC concentration for all the test pathogens representing antibiofilm activity of *N. nucifera* methanolic crude extract against test organisms.

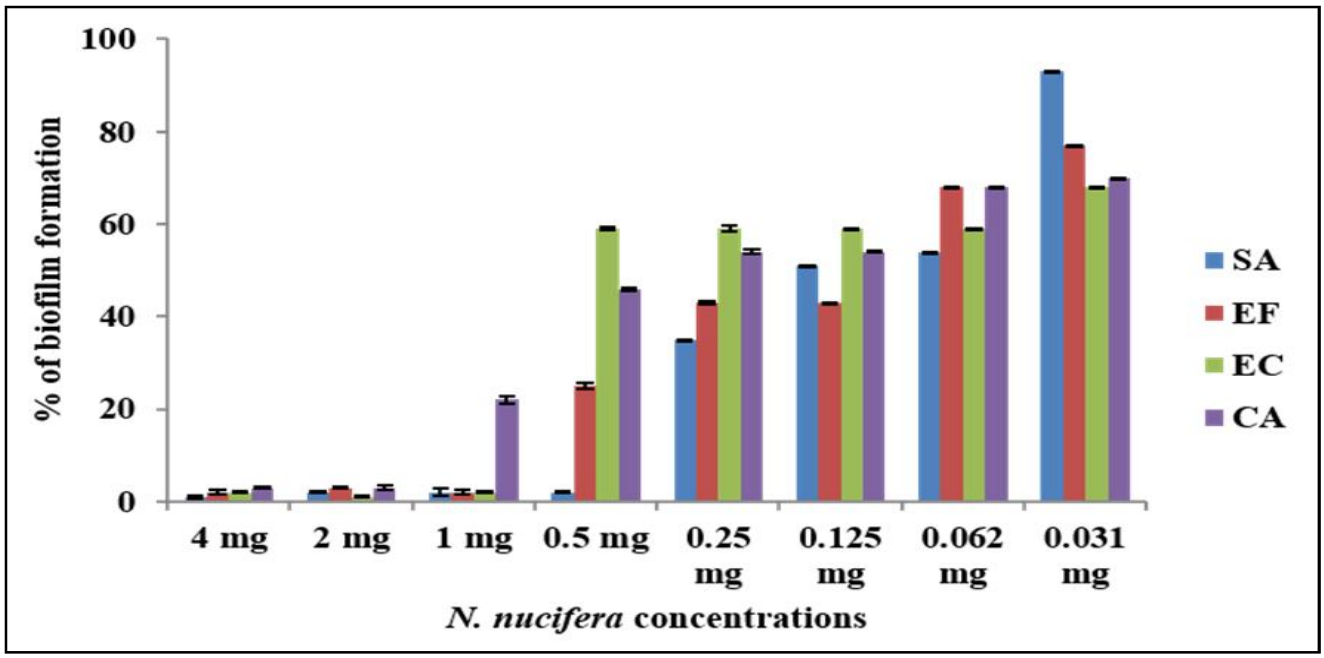


Figure 3: *N. nucifera* methanolic crude extract impact on the biofilm-forming capacity of *E. coli, S. aureus, C. albicans,*and *E. faecalis.* Note: SA-*S. aureus,* EF- *E. faecalis,* EC- *E. coli* and CA-*C. albicans*.

3.4 Effect of *N. nucifera* crude methanolic extract on mature biofilm eradication

The effect of *N. nucifera* methanolic crude extract on mature biofilms formed by *E. coli, C. albicans, S. aureus,* and *E. faecalis* on non-living surfaces was quantified, and the results are shown in Figure 4. Here, the *N. nucifera* methanolic crude extract effectively eliminated all the test pathogens' biofilm after treatment with various concentrations. The 3X, 2X, and 1X MICs of *N. nucifera* methanolic crude extract eliminated 78%,79%, and 83% of *S. aureus* mature biofilm, respectively. In the same way, *E. faecalis* 74%, 82%, and 87% biofilm elimination after 1X, 2X, and 3XMIC concentrations treatment. Similarly, 72%, 79%, and 83% of *E. coli* mature biofilm were eliminated after treatment, and 70%,77%, and 89% of *C. albicans* mature biofilm were effectively eliminated by *N. nucifera* methanolic crude extract, which indicates the antibiofilm potential of the extract against the selected microorganisms.

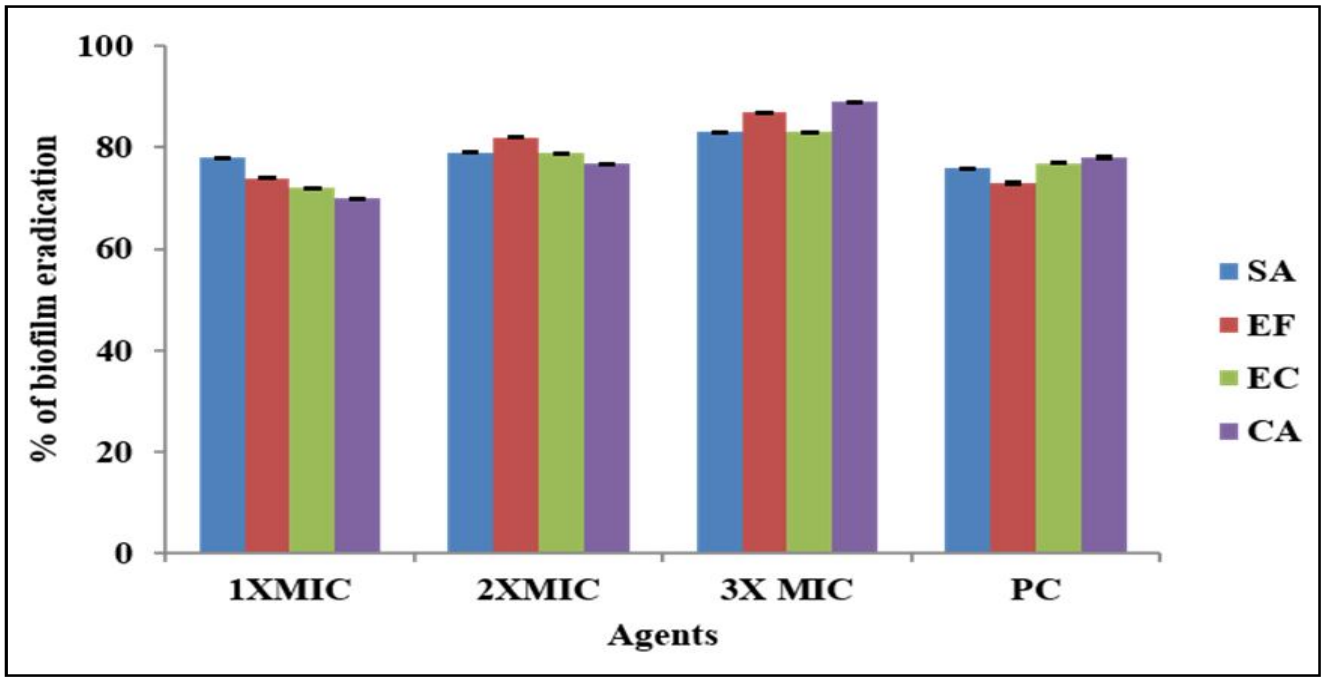
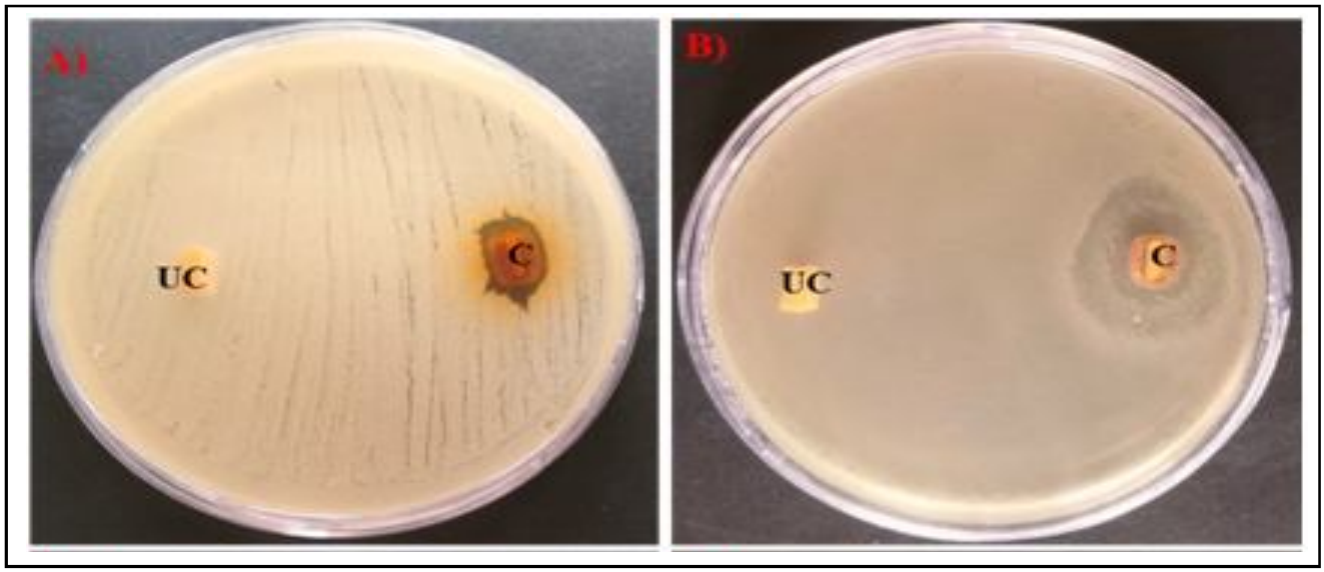


Figure 4: Quantitative analysis of *N. nucifera* methanolic crude extract impact on *E. coli, S. aureus, C. albicans,* and *E. faecalis* mature biofilms. Note: SA-*S. aureus*, EF- *E. faecalis*, EC- *E. coli* and CA-*C. albicans*.Note: PC- Positive controls.

3.5 Antimicrobial potential of*N. nucifera* methanolic crude extract coated catheter

The *N. nucifera* methanolic crude extract coated catheter tube antimicrobial potential investigated against *S. aureus, E. coli, C. albicans,* and *E. faecalis* using *an in vitro* bladder modelis displayed in Figure 5. The clear growth inhibition surrounding the catheter tube represents the antimicrobial activity of *N. nucifera* methanolic crude extract hereby the anti-adhesive property was proved.



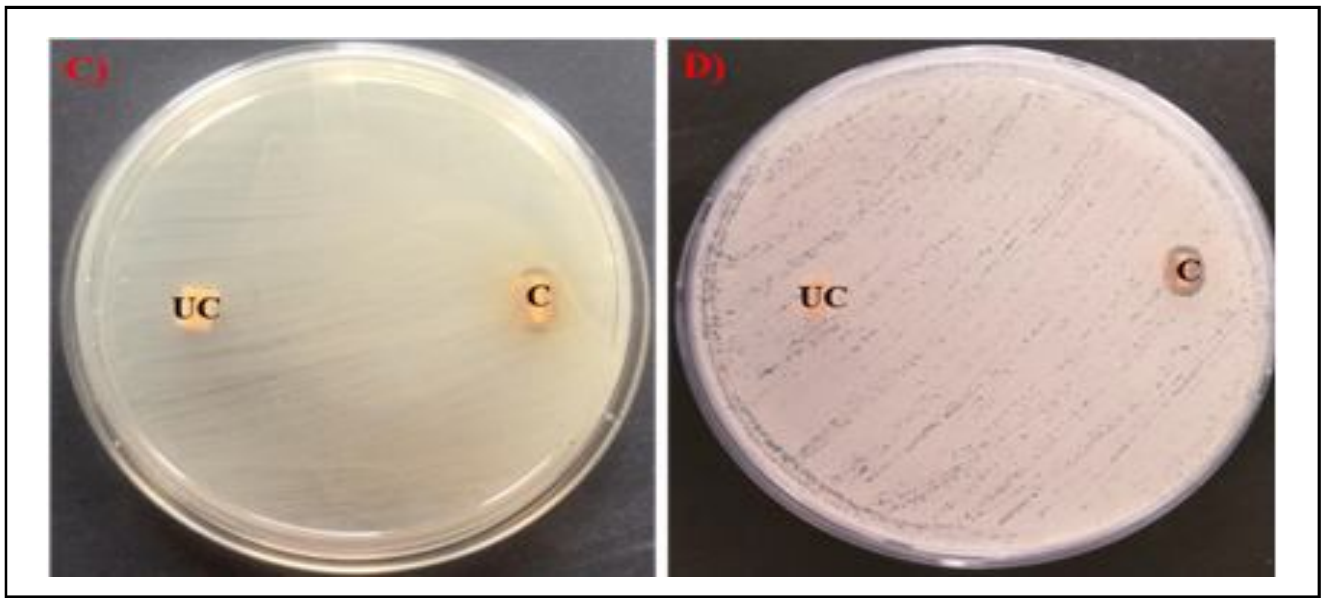


Figure 5: *N. nucifera* methanolic crude extract coated catheter tube antimicrobial activity against *E. coli, S. aureus, C. albicans,* and *E. faecalis* using *in vitro* bladder model. (A) *S. aureus,* (B) *E. faecalis,* (C) *E. coli,* and (D) *C. albicans.* Note: UC- uncoated, C- coated with *N. nucifera* extract.

3.6 Antioxidant property of *N. nucifera* methanolic crude extract

The *N. nucifera* methanolic crude extract antioxidant property was evaluated by DPPH and the calculated free radical scavenging percentage is plotted in Figure 6. The figure shows the different concentrations of *N. nucifera* methanolic crude extract such as 4 mg/ml, 3 mg/ml, 2 mg/ml, 1 mg/ml and 0.5 mg/ml showed 26%,42%,47%, 57%,65% and 83% of free radical scavenging activity, respectively.

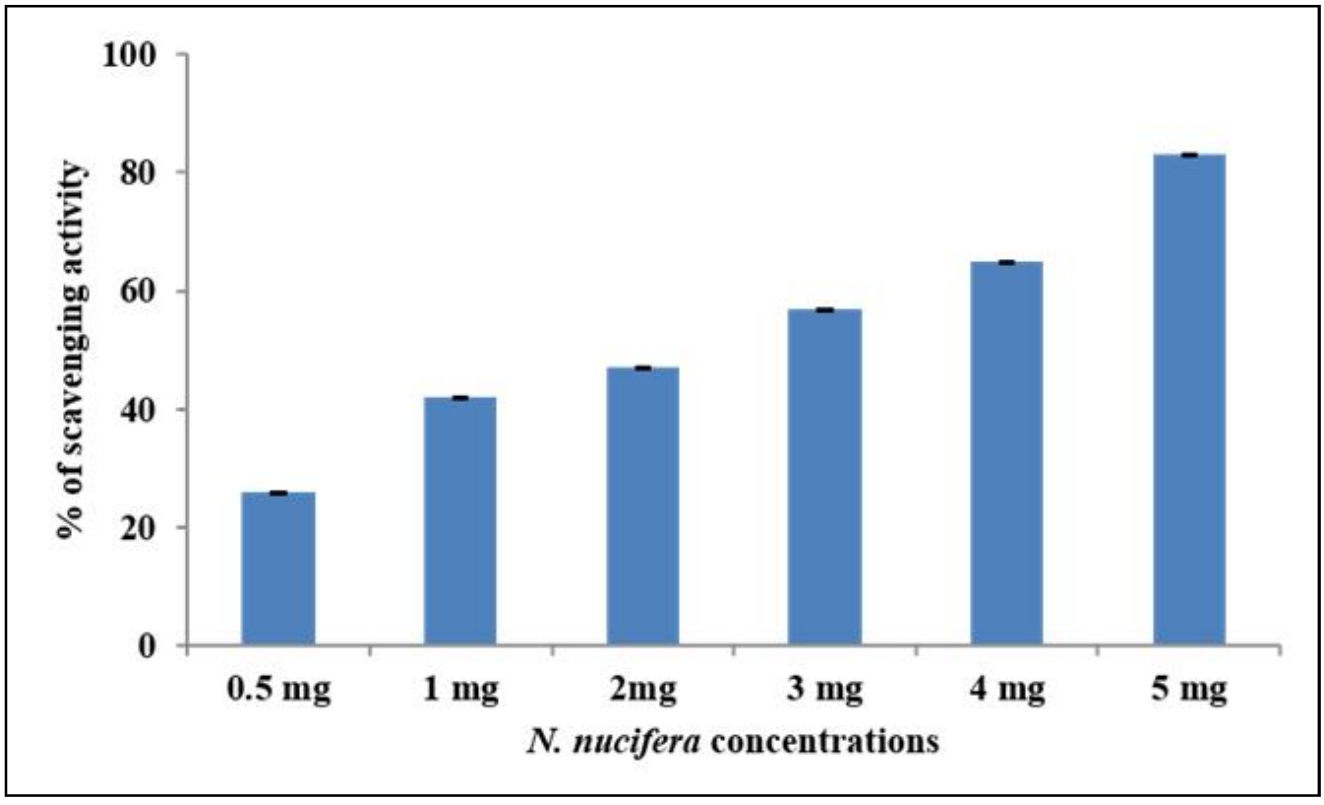


Figure 6: *N. nucifera* methanolic crude extract antioxidant property was evaluated by DPPH.

3.7 Cytotoxicity of *N. nucifera* methanolic crude extract

The *N. nucifera* methanolic crude extract cytotoxicity predicted against L_{929} cells is presented in Figure 7. As observed in the figure, the graph manifested the various concentrations of *N. nucifera* methanolic crude extract treated L_{929} cells viability was denoted. The maximum cell viability of 86% was noted at 0.5 mg/ml of crude extract when compared with untreated cells and thus, the *N. nucifera* methanolic crude extract was not cytotoxic.

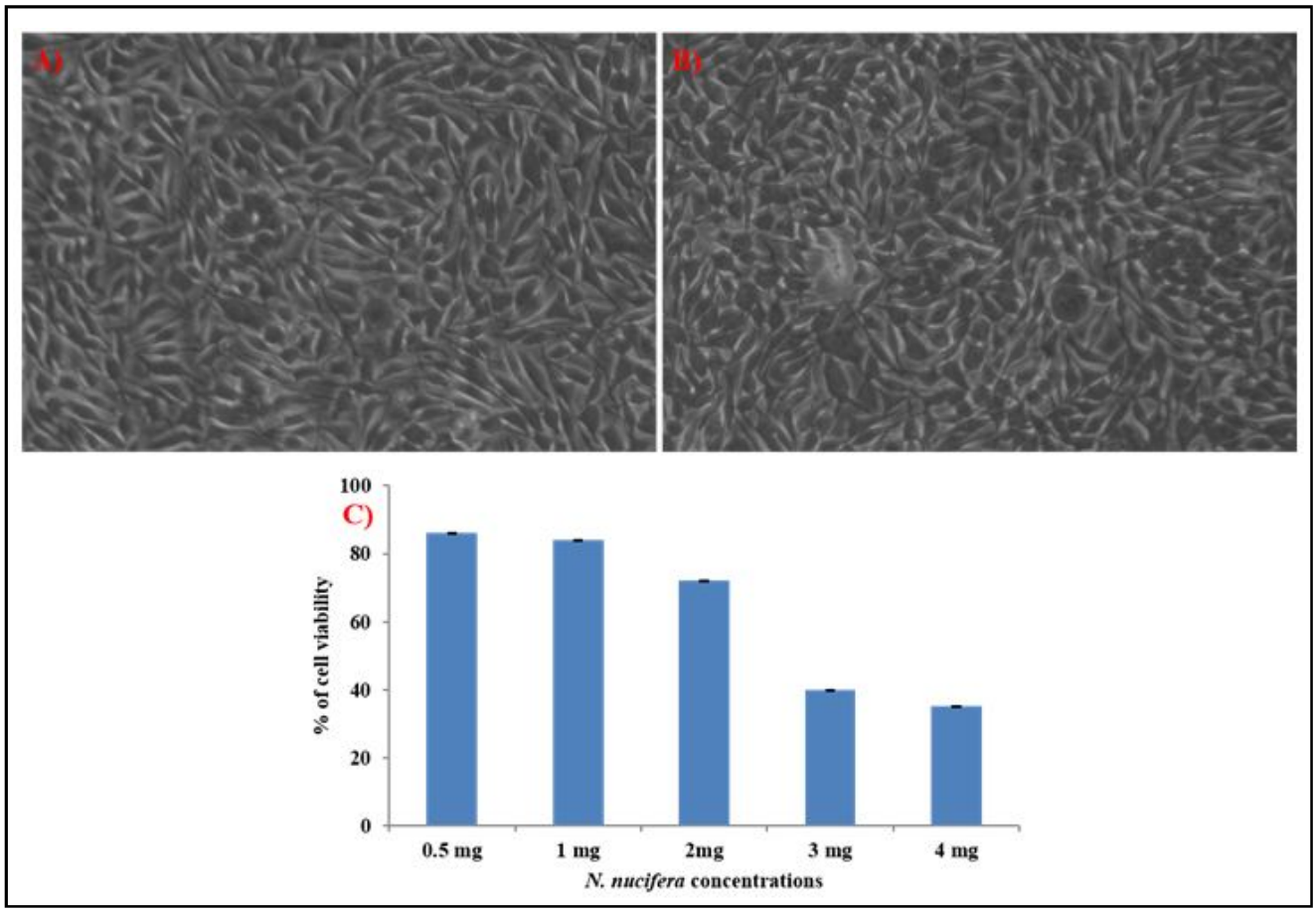


Figure 7: ***N. nucifera*** **methanolic crude extract cytotoxicity on L_{929} cells. (A) Untreated cells, (B) Treated with** ***N. nucifera*** **methanolic crude extract, (C) Graph denotes the cell viability percentage after treatment with various concentrations of** ***N. nucifera.***

4. Discussion

The multidrug-resistant biofilm-forming organisms are mostly responsible for significant nosocomial infections such as CAUTI which is one of the important medical devices associated with infection resulting in high mortality and morbidity due to drug-resistant and biofilm-forming ability. Therefore, urgent drug development with potential antimicrobial and antibiofilm activity is needed to fight against microbes. Hence, the present study investigated the *N. nucifera* methanolic crude extract antimicrobial potential against *S. aureus, E. coli, C. albicans,* and *E. faecalis* and evidenced antimicrobial activity against test pathogens with minimal inhibitory concentrations. Supporting the findings of the present investigation, a study reported the antipathogenic activity of *N. nucifera* (lotus plant) leaf extracts were prepared through various solvents including acetone, hexane, and methanol against *Bacillus subtilis* and *C. albicans*. Among the solvents, the methanolic leaf extract exhibited extreme antibacterial activity against *B. subtilis*. In contrast, hexane and acetone-extracted solvents demonstrated maximum antifungal activity against *C. albicans,* indicating antimicrobial activity of all solvent extracts (Techaoei *et al*., 2020; Arjun *et al*., 2012).

Besides the antimicrobial activity, *N. nucifera* was also investigated for its antibiofilm potential against the test microorganisms. The catheterization process allows microbial entry through the catheter lumen from the external environment leading to biofilm formation through multiple steps such as attachment, colony formation, and maturation have complex structure that makes treatment challenges for clinicians (Pelling *et al*., 2019; Zhu *et al*., 2019). Therefore, our idea was to focus on every stage of biofilm formation on non-living surfaces. The present study investigated the antibiofilm activity of *N. nucifera* crude extract against test pathogens and found the activity by biofilm formation inhibition and also, eradicating five days mature biofilms thereby antibiofilm activity was proved. Additionally, the biofilm formation was started through the catheter lumen when the microbes entered which makes treatment crucial. Therefore, the coating of the catheter's inner and outer with methanolic extract of *N. nucifera* is an excellent method for biofilm eradication on the

catheter surface. Hence, our study examined the antimicrobial activity of *N. nucifera*-coated catheter against test pathogens and found better activity by forming a zone around the catheter. In support of this, several studies reported coating of the catheter with various antipathogenic compounds including polymers, zinc oxide, some antibiotic combinations, silver, and fosfomycin and their activity towards tested organisms such as *E. faecalis*, *E. coli*, *P. aeruginosa*, *K. pneumoniae* and *C. albicans* (Ivanova *et al*., 2021; Jia *et al*., 2021; Rahuman *et al*., 2021; Abbott *et al.,* 2020; Fisher *et al*., 2015). Overall, our results suggested that *N. nucifera* the methanolic extract exposed excellent antimicrobial and antibiofilm activity *in vitro*, and further studies are needed to conclude the practical application of coating catheters for CAUTI infection.

5. Conclusion

Uropathogens cause several diseases, including catheter-associated urinary tract infections (CAUTIs), which, when caused by biofilm-forming multidrug-resistant microorganisms, would pose management and treatment challenges. As the researchers are investigating potential phytocompounds that can be developed as antibiofilm catheter coatings. The present study analyzed the antibiofilm and antimicrobial potentials of the methanolic extract of a plant - *N. nucifera* against selected CAUTI-causing microorganism. The extract showed remarkable antiactivities against all the selected pathogens and it could successfully eradicate the biofilms formed by these organisms. Also, the *in vitro* bladder model analysis showed that the catheters coated with the plant extract could prevent the formation of biofilms when exposed to these pathogens. As the plant extract was proved to be noncytotoxic, the authors recommend further detailed *in vivo* analyses of the *N. nucifera* to make it available as a catheter coating agent to control CAUTIs.

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Conflict of interest

The authors declare no conflicts of interest relevant to this article.

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