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Antibacterial, antifungal, antibiofilm and antioxidant properties of *Orthosiphon stamineus* Benth.: An *in vitro* analysis

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Abstract

Indwelling medical devices have been playing a major role in healthcare settings in providing health support for patients. Among the several medical devices, catheter insertion gained much attention due to their infectious nature by allowing microbial entry and colonization on their surfaces resulting in biofilm formation which makes the treatment more critical. Therefore, alternative antimicrobial agents are urgently needed to avoid such a situation, and plant-based natural biomolecules have always been attractive owing to plenty of health benefits. Hence, our study investigated the antimicrobial potential of the methanolic crude extract of *Orthosiphon stamineus* Benth. Some of the microbes causing catheter-associated urinary tract infections, like *Candida albicans*, *Staphylococcus aureus*, *Escherichia coli*, and *Enterococcus faecalis*. The antimicrobial potential was analyzed by well diffusion method and the minimal inhibitory concentrations (MICs) were found to be 0.312 mg/ml against each one of the selected pathogens. The antibiofilm activity was studied using the crystal violet method and it was found that the extract could effectively inhibit the formation of biofilms when applied with different concentrations of *O. stamineus*. The antibiofilm activity was quantified against mature biofilms after treatment with various concentrations and the extract reduced the biofilms by 87%, 86%, 88%, and 88% in the cases of *S. aureus*, *C. albicans*, *E. faecalis*, and *E. coli,* respectively. The *in vitro* bladder model revealed the antimicrobial potential of *O. stamineus* coated catheters against the test pathogens. In addition, *O. stamineus* crude extract showed potent antioxidant properties using DPPH assay and also had no cytotoxicity on L929 cells. Thus, the phytocompounds in the plant *O. stamineus* need to be further purified and studied in detail for their use as an alternative agent for CAUTI management.

1. Introduction

In the human body, the urinary system has a significant role in excreting liquid waste and keeping the individual healthy, in general, the urinary tract is sterile as it flushes out liquid waste and prevents bacterial entry into the bladder or urethra (Andersen *et al*., 2020; Feneley *et al*., 2015). More importantly, in healthy individuals, if the bacteria enter into the bladder, they will be expelled during urination, and in any circumstances, the liquid waste remains in the bladder, and the interior bladder surfaces resist bacterial adherence resulting in no colony formation (Yisiak *et al*., 2021; Flores-Mireles *et al*., 2019). In inevitable situations, indwelling medical devices such as urinary catheters are one of the most important techniques used in the modern medical field to support the healthcare of the particular individual (Cortese *et al.,* 2018; Gould, 2015). Unfortunately, the long-term usage of urinary catheters makes the catheters prone to infection by allowing microbial entry into the urinary tract through the drainage system resulting in catheter-associate urinary tract infections (CAUTIs) and are responsible for 40% of nosocomial infections creating high mortality and morbidity rates (Werneburg, 2022; Milo *et al*., 2019; Haque *et al*., 2018; Hollenbeak *et al*., 2018). CAUTIs lead to complications like endotoxic shock, pyelonephritis, bladder stones, catheter encrustation, and septicemia (Gallingani *et al*., 2023; Citla *et al*., 2020; Cortese *et al*., 2018; Jacobsen *et al*., 2008). CAUTIs are caused by a variety of microbes including bacteria and fungi such as *Candida albicans, Staphylococcus aureus, Escherichia coli,* and *Enterococcus faecalis* (Najla *et al*., 2023; Kim *et al*., 2017; Sharma *et al*., 2016; Chatterjee *et al*., 2014). CAUTI-causing microorganisms initially colonize the catheter surface and produce extracellular polymeric substances which help the cells to form biofilm. These biofilms are complex three-dimensional structures composed of a protective polymeric matrix which can decrease the antibiotic effectiveness through various mechanisms including horizontal gene transfer, efflux pumps, and target site alteration resulting in resistant microbes making treatment ineffective (Aleksandra *et al*., 2020; Kurmoo *et al*., 2020; Sandhu *et al*., 2018). So, there is a need for alternate antimicrobial agents.

For centuries, natural resources such as plants, marine organisms, and soils have been gaining huge attraction owing to the presence of a varied range of novel biomolecules along with different pharmacological properties. Among the several natural sources, plant-based natural biomolecules have gained much attention due to the secondary metabolites production in the plants and they provide various health benefits along with potential bioactivities such as antimicrobial, anticancer, antioxidant, *etc*. *O. stamineus* is one of the most significant medicinal plants used in Chinese folk medicine and extensively distributed in many countries across the world, its use

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has been known since time unknown in the treatment of kidney diseases and diabetes. Several studies have mentioned the pharmacological properties of *O. stamineus* like antibacterial, antioxidant, antidiabetic, and anti-inflammatory properties (Wang *et al*., 2022; Li *et al*., 2017; Ma *et al.,* 2015). Unfortunately, limited studies are reported on the antimicrobial potential of the plant against pathogenic organisms causing CAUTIs. Therefore, this study investigates the antibiofilm and antiadhesive effect of *Orthosiphon stamineus* against *S. aureus, C. albicans, E. faecalis,* and *E. coli* which are frequently isolated organisms in CAUTI.

2. Materials and Methods

2.1 Inoculum preparation

Overnight cultures of *S. aureus, C. albicans, E. faecalis,* and *E. coli* (procured from Microbiologique, USA with ATCC (American Type Culture Centre) numbers 12600, 2091, 14506, and 13706 respectively) were adjusted to standard level (0.5 MacFarland unit) and were used for all the studies in the present investigation. The Mueller Hinton broth (MHB), BHI (Brain Heart Infusion), and Sabouraud Dextrose Broth (SDB) which were procured from HiMedia Laboratories Private Limited, India were used to grow the selected organisms. The analyses were done in triplicates and ampicillin, rifampicin, and nystatin were used as positive controls, and methanol was used as the vehicle control.

2.2 *O. stamineus* crude extract preparation

O. stamineus plant was collected from the nursery at Deseeya Ayurvedic Pharmacy, Kozhikode, Kerala, India, and was authenticated (No. DAP/MPA-25/2024) as per the standard protocols by Dr. Shamna, Technical Supervisor at the factory. To prepare the plant's crude extract, 20 g of the leaf powder was filled in cellulose thimble and left in the Soxhlet apparatus as per standard procedure (Harley *et al.,* 2022). The solvent methanol was poured in the required volume into the flask and the process was started after setting temperature at 60°C. The reaction was continued until the colored solutions turned into a colorless solution. The solvent was evaporated and the final product was used further for all the studies.

2.3 Antimicrobial activities of *O. stamineus* crude extract

Using well diffusion method as per the standard protocols (Meiyazhagan *et al*., 2016), the antimicrobial potential of the O. *stamineus* crude extract against *S. aureus, C. albicans, E. faecalis,* and *E. coli* was studied. The wells were drilled after swabbing the overnight cultures of all the test pathogens on specified sterile plates and the wells received various concentrations (ranging between 1.25 mg/ml and 0.009 mg/ml) of *O. stamineus* and incubated. The antimicrobial efficiency of *O. stamineus* crude extract was found after observing the clear zone formation around the well against test pathogens.

2.4 Minimal inhibitory concentration (MIC) determination of *O. stamineus* crude extract

The microdilution method determined the *O. stamineus* crude extract MIC against *S. aureus, C. albicans, E. faecalis,* and *E. coli* (Meiyazhagan *et al*., 2015). The serial dilution was done for 1.25 mg/ml of *O. stamineus* concentration to reach 0.009 mg/ml in the respective broth and the overnight cultures of all the test pathogens were added to the plates and were incubated. The MIC was determined after analyzing the OD at 600 nm.

2.5 Effect of *O. stamineus* crude extract on biofilm formation

The assay using crystal violet (Meiyazhagan *et al*., 2015) was used to investigate the activity of O. *stamineus* crude extract on the biofilm formation by *S. aureus, C. albicans, E. faecalis,* and *E. coli*. For the assay, the biofilm formation of all the test pathogens was observed for 5 days with different concentrations of (1.25 mg/ml, 0.65 mg/ml, 0.312 mg/ml, 0.156 mg/ml, 0.078 mg/ml, 0.039 mg/ml, 0.019 mg/ml and 0.009 mg/ml) *O. stamineus*. The attached biofilms were methanol-fixed after washing followed by crystal violet staining of biofilms. The purple-colored final compound was measured at 570 nm after destaining with an ethanol-acetone mixture.

2.6 *O. stamineus* crude extract effect on mature biofilm

To quantify the antibiofilm efficiency of the crude extract of *O. stamineus* on mature biofilms of *S. aureus, C. albicans, E. faecalis,* and *E. coli,* the crystal violet assay (Meiyazhagan *et al*., 2015) was performed. The effect of three different concentrations (1X, 2X, and 3X MIC) of *O. stamineus* was studied on a 5-day-old biofilm for 24 hrs. The adherent biofilms were washed after treatment and fixed with methanol subsequently crystal violet staining. The ethanol-acetone solution mixture was poured to destain and the final product was measured for its optical density at 570 nm.

2.7 Antimicrobial activities of catheters coated with *O. stamineus* extract

Using an *in vitro* bladder model, the antibacterial and anticandidial potential of catheters coated with *O. stamineus* extract was evaluated against *S. aureus, C. albicans, E. faecalis,* and *E. coli* (Goda *et al.,* 2022). In short, sterile pieces of catheters were coated with *O. stamineus* crude extract and subjected to air-drying. Then, they were placed over sterile respective plates which were swabbed with appropriate test pathogens and kept for incubation. The formation of growth inhibition zones around the extract-coated catheter pieces indicated the antimicrobial activities of the extract against the pathogenic microbes.

2.8 Antioxidant properties of *O. stamineus*

Free radical scavenging assay (Gayathri and Sathish, 2016) using DPPH (2, 2-diphenyl-1-picryhydrazyl) was performed to evaluate for *O. stamineus* antioxidant property. Different *O. stamineus* concentrations were combined with DPPH solution for 30 min and the final reactions were read at 517 nm. The radical scavenging activity of *O. stamineus* percentage was calculated as shown:

Scavenging effect = 100 × (blank OD – sample OD)/ blank OD

2.9 Toxicity of *O. stamineus* extract on mammalian cells

O. stamineus crude extract was investigated for its effect on mammalian cells (L_{929}) using MTT assay (Meiyazhagan *et al*., 2015). The crude extract of *O. stamineus* was added in various concentrations to treat L_{929} cells and was grown on DMEM (Dulbecco's Modified Eagles Medium) with fetal bovine serum (10%) and incubated. 20 µl of MTT solution (5 mg/ml in PBS) were added to form formazan product and the final purple-colored compound was measured at 570 nm and 690 nm after adding DMSO. The cytotoxic effect of *O. stamineus* crude extract was calculated as mentioned below:

Cell viability percentage = [(Treated cells OD)/ (Untreated cells OD)] × 100.

2.10 Statistical analysis

For all the experiments, mean and standard deviations were performed to calculate the standard error.

3. Results

3.1 *O. stamineus* crude extract antimicrobial activity

The antimicrobial activities of *O. stamineus* crude extract studied against *S. aureus, C. albicans, E. faecalis,* and *E. coli* using well diffusion method is presented in Figure 1. The antimicrobial activities were indicated by around clear zone formation of the well against all the test pathogens. The activities were increased when the extract's concentration increased as in the figure, and the fact hints that the antimicrobial potential of *O. stamineus* is concentration-dependent.

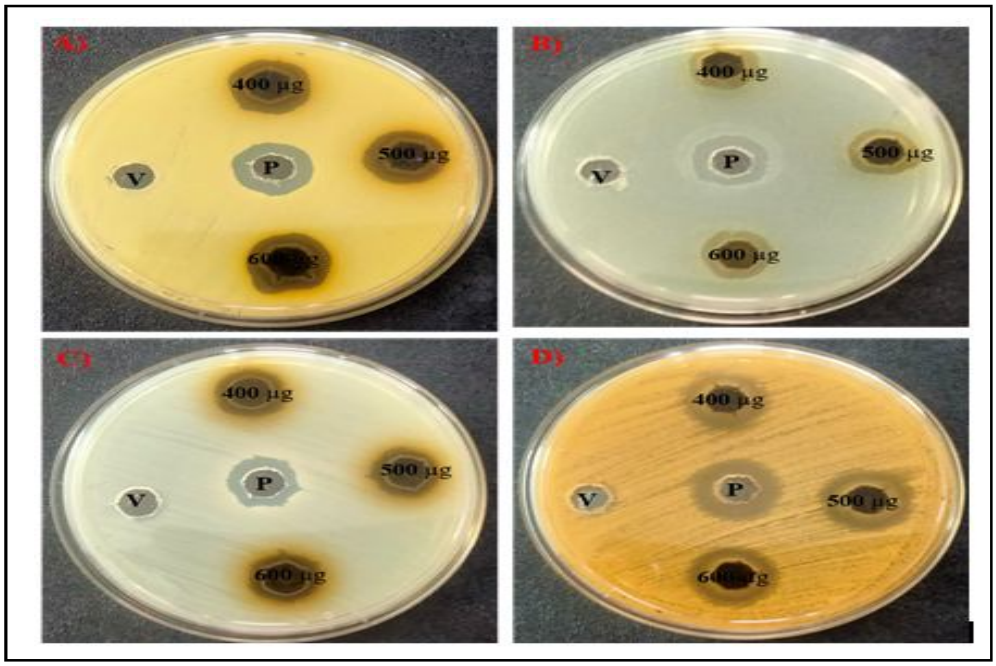

Figure 1: Antimicrobial activity of *O. stamineus* against: *A. S. aureus,* B. *E. faecalis,* C. *E. coli* and D. *C. albicans*. Note: V-vehicle control and P- positive controls.

3.2 MIC determination of *O. stamineus* crude extract

The *O. stamineus* extract was determined for its MICs against *S. aureus, C. albicans, E. faecalis,* and *E. coli* and the findings are displayed in Figure 2. The minimal concentrations of *O. stamineus* needed for pathogenic growth inhibition are clear in the graph and were 0.312 mg/ml against all the test pathogens.

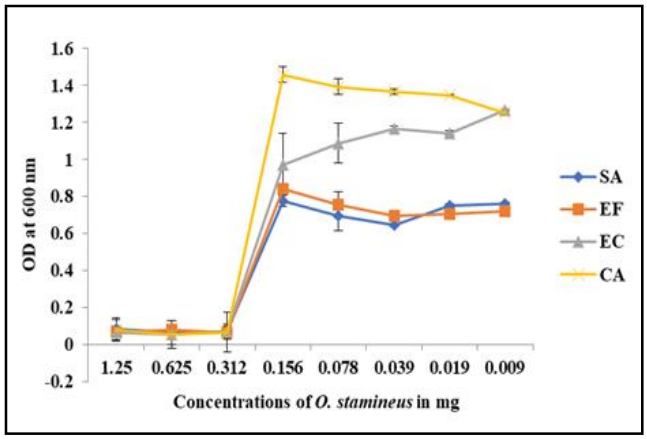

Figure 2: Minimum inhibitory concentration determination for *O. stamineus* extract against *S. aureus, C. albicans, E. faecalis,* and *E. coli.*

3.3 Effect of *O. stamineus* crude extract on the formation of biofilms

The effect of *O. stamineus* crude extract on biofilm formation by *S. aureus, C. albicans, E. faecalis,* and *E. coli* was studied using the crystal violet method. The findings are shown in Figure 3. The percentage of biofilm formation after treatment with varying concentrations of *O. stamineus* was calculated. The extract showed effective inhibition of growth at its MIC level against all the test pathogens. Moreover, the biofilm formation was gradually increased after the MIC level.

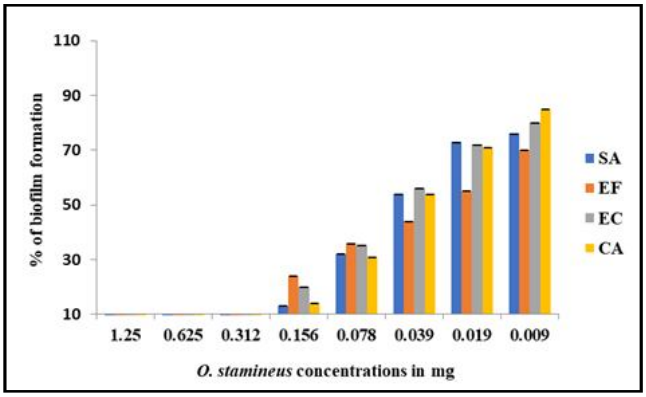

Figure 3: Effect of *O. stamineus* crude extract on formation of biofilms by *S. aureus, C. albicans, E. faecalis,* and *E. coli.*

3.4 Quantification of the efficiency of *O. stamineus* crude extract on biofilm formation

The efficiency of *O. stamineus* crude extract on biofilm formation was quantitatively analyzed and the findings are presented in Figure 4. The percentage of biofilm eradication after treatment with 1X MIC, 2X MIC, and 3X MIC of *O. stamineus* showed effective reduction against all the test pathogens. The *O. stamineus* effectively reduced a maximum of 88% biofilm eradication after 3X MIC treatment against *S. aureus* and *E. faecalis*. Similarly, 87% and 86% of biofilm reduction were observed after 3X MIC treatment against *C. albicans* and *E. coli* representing the potential antibiofilm activity of *O. stamineus* extract.

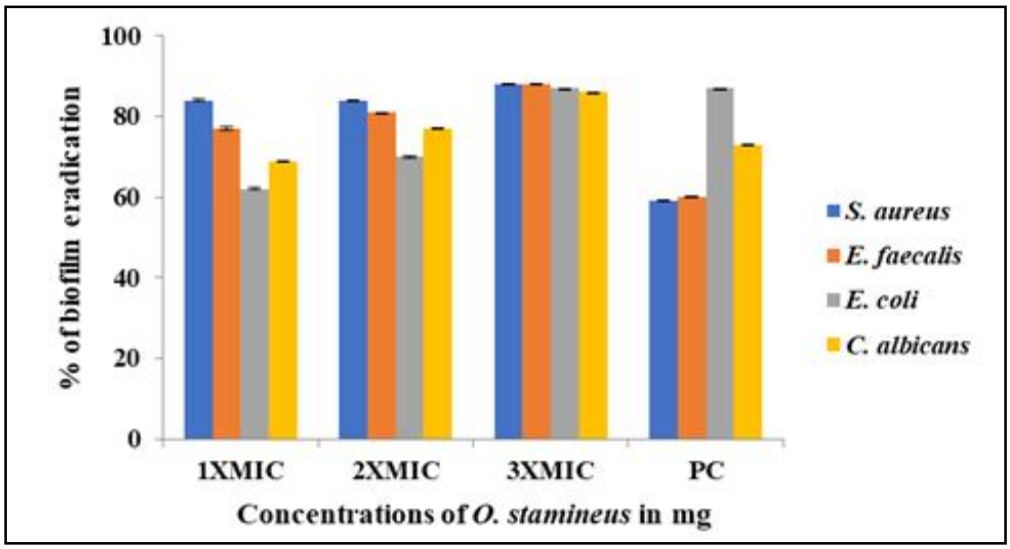

Figure 4: Quantification of antibiofilm activity of *O. stamineus* against test pathogens.

3.5 Antimicrobial activity of catheter coating with *O. stamineus*

The antimicrobial potential of *O. stamineus* extract-coated catheter was analyzed using an *in vitro* bladder model against *S. aureus, C. albicans, E. faecalis,* and *E. coli* and the result is presented in Figure 5. The clear zones were developed around the tubes and they indicated the antimicrobial activities of *O. stamineus*-coating on the test pathogens.

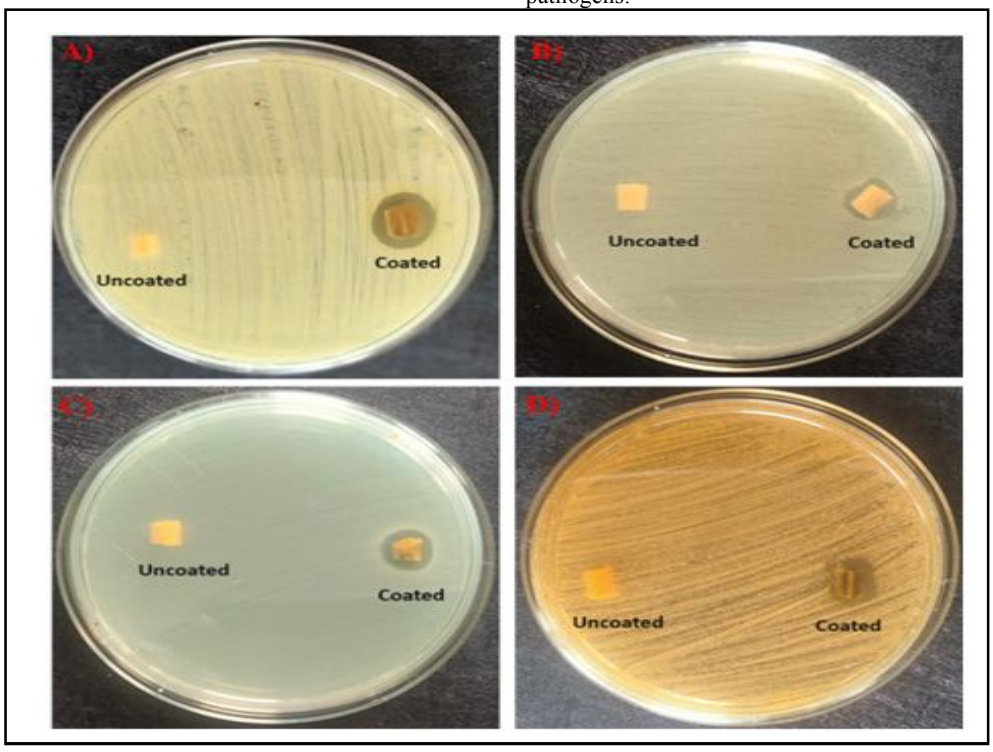

Figure 5: Antimicrobial activity of *O. stamineus* crude extract-coated catheter on *in vitro* bladder model by A. *S. aureus*, B. *E. faecalis*, C. *E. coli*, and D. *C. albicans*.

3.6 Antioxidant properties of *O. stamineus* extract

The antioxidant potential of *O. stamineus* extract was analyzed using DPPH (2,2-Diphenyl-1-picrylhydrazyl) and the findings are shown in Figure 6. The calculated free radical scavenging activity of various concentrations of *O. stamineus* showed a maximum of 71% inhibitory activity indicating potential antioxidant properties.

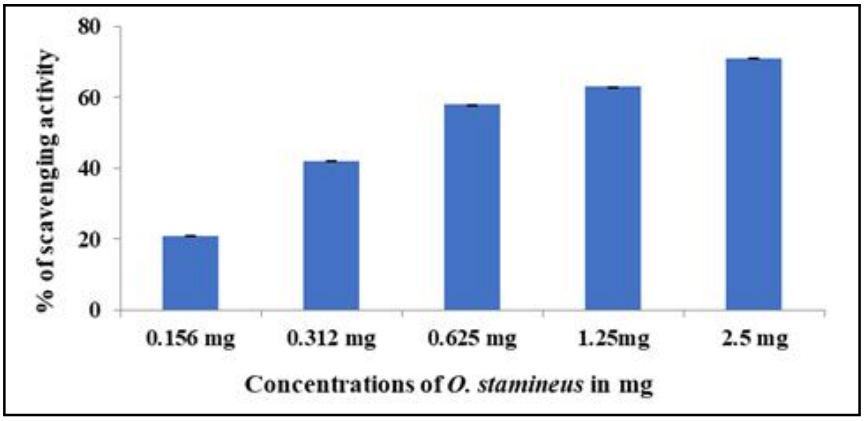

Figure 6: Antioxidant properties of *O. stamineus* crude extract.

3.7 *O. stamineus* effect on mammalian cells

The effect of *O. stamineus* extract on L_{929} cells was studied to find out the cell viability percentage against various concentrations of *O. stamineus*, and the results are presented in Figure 7. The *O. stamineus* extract induced no toxicity to normal cells when treated with MIC level but slight toxicity was noticed after in high concentration when compared to untreated cells.

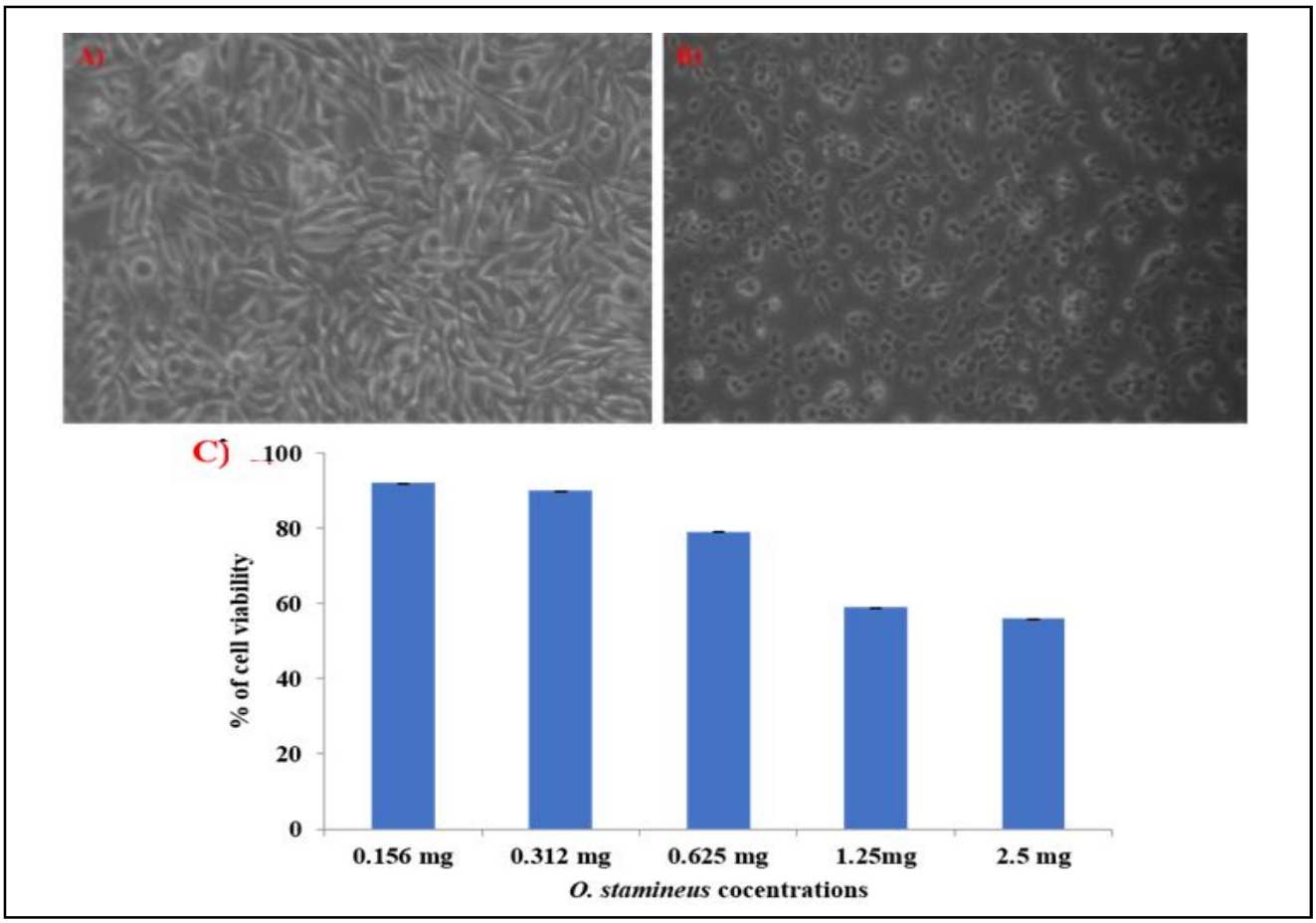

Figure 7: Effect of *O. stamineus* on L_{929}. A. Untreated cells, B. Cells treated with *O. stamineus* crude extract, and C. Graph representing the cell viability obtained after treatment with different concentrations of *O. stamineus* extract (20X magnification).

4. Discussion

Catheter-associated urinary tract infections are caused by infections on indwelling urinary catheters and are among the most important hospital-acquired infections. In general, CAUTIs are mainly related to biofilms which make the treatment challenging as a result of the development of multidrug resistance in causative pathogens. Therefore, the need for new, novel antimicrobial agents that may combat drug resistance and eliminate the biofilm on the catheter surface is urgent. The plant-based natural compounds have gained much attraction owing to plenty of health benefits. So, the present study investigated the methanolic crude extract of *O. stamineus* for its antibiofilm and antimicrobial activities against *S. aureus, C. albicans, E. faecalis,* and *E. coli* which are frequently isolated from CAUTI cases. The results showed potent antimicrobial activities with minimal inhibitory concentrations against test pathogens. In support of this, a recent study investigated the antimicrobial potential of *O. stamineus*-derived oil against *Streptococcus mutans, Streptococcus mitis, Aggregatibacter actinomycetemcomitans, E. faecalis, Fusobacterium nucleatum, S. aureus, Porphyromonas gingivalis,* and *Streptococcus salivarius* which are commonly involved in dental infections and, the plant oil showed potent inhibitory as well as killing kinetics after treatment against all the test pathogens. The compound induced significant alteration in the cell morphology was noted due to the phytochemicals such as eugenol and humulene present in the compound indicating it has a very good effect on Gram-positive organisms (Azizan *et al*., 2017). Similarly, another study (Ho *et al.,* 2010) investigated the antibacterial and antioxidant properties of various propositions of methanolic crude *O. stamineus* extract and found a potent antibacterial effect against food-borne pathogen *Vibrio parahaemolyticus.* Interestingly, they found the antibacterial effect was in the presence of rosmarinic acid which also showed excellent antioxidant properties. In another research (Deipenbrock *et al*., 2023), the *ex vivo* activity of *O. stamineus* was analyzed against uropathogenic *E. coli*, and antiadhesive activity was studied in bladder and kidney lesions in a mice infectious model. The *O. stamineus* was combined with caffeic acid, rosmarinic acid, and cichoric acid produced significant reproducibility and it explained the anti-infective and antiadhesive effect of *O. stamineus* against *E. coli.* Romulo *et al*. (2018) investigated the antimicrobial activities of *Orthosiphon aristatus,* another member from the same genus against *E. faecalis, E. coli, C. albicans, S. aureus,* and *Pseudomonas aeruginosa* and found potent anticandidal activity indicating *O. aristatus* can be a better antifungal agent and it also prevented the kidney and bladder infection *in vivo* (Sarshar *et al*., 2017).

In addition to the antibacterial effect, the *O. stamineus* crude extract was analyzed for its antibiofilm activity against test pathogens. The biofilm-related CAUTI played an important role in nosocomial infection. The biofilm formation has several steps including attachment, colonization, and maturation. The present study aimed to focus on every step of biofilm formation and found potent antibiofilm activity by inhibiting biofilm formation and reducing the biofilms of all the test pathogens. Additionally, to avoid the catheter surface biofilm, the coating of the catheter with *O. stamineus* crude extract could be an excellent alternative method, if developed by further investigations. Therefore, the *in vitro* bladder model provides the better antimicrobial activity of *O. stamineus* against all the test pathogens. Similarly, various studies provide the different antimicrobial agents such as silver, zinc oxide, antibiotic combination, and polymer, and also, fosfomycin which showed excellent antimicrobial activity against *P. aeruginosa, E. faecalis, E. coli,* and *S. aureus (*Aleksandra *et al*., 2021; Jia *et al*., 2021; Rahuman *et al*., 2021; Abbott *et al*., 2020; Fisher *et al*., 2015).

Besides the antibacterial and antibiofilm activities, *O. stamineus* crude extract was analyzed for its antioxidant properties and the study revealed the potent antioxidant properties of *O. stamineus.* Similarly, various solvent extracts of *O. stamineus* were analyzed for antioxidant properties and revealed the potent antioxidant properties due to the presence of andrographolide and rosmarinic acid indicating it may be better applied in human health (Malahubban *et al*., 2013). In addition. The compound is intended for human application, the *O. stamineus* crude extract was analyzed for its cytotoxic effect on mammalian human cells and showed no cytotoxic effect which indicates *O. stamineus* biocompatibility.

5. Conclusion

The crude methanolic extract of *O. stamineus* was analyzed for its antimicrobial activities against *S. aureus, C. albicans, E. faecalis,* and *E. coli* and showed excellent antibacterial effects with minimal concentration needed for test pathogen growth inhibition. The antibiofilm activity revealed potent biofilm inhibition and effectively eradicated mature biofilm. The catheter coating revealed excellent antimicrobial activity in the *in vitro* bladder model which mimics suitable environmental conditions. It also had anti-oxidant properties and it was cytotoxic to mammalian cells. At the same time, the study has certain limitations like, it was just an *in vitro* analysis and, the purification of phytochemical compounds responsible for the antimicrobial activities was not performed. Based on the findings of this study, the authors recommend that *O. stamineus* plant may be further purified for its phytochemicals responsible for the antimicrobial activities and developed for its clinical applications against CAUTI-causing microorganisms.

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Conflict of interest

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

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