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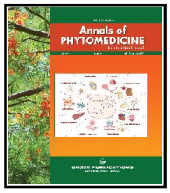

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Pharmacological evaluation of *Kalanchoe pinnata* (Lam.) Pers. methanolic extract: An *in vitro* investigation for antioxidant and antibacterial properties

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Abstract

Catheter-associated urinary tract infections (CAUTIs) are caused by antibiotic-resistant and biofilm-forming organisms, which gained much importance due to the complications in hospitalised patients that make treatment difficult. Therefore, urgent drug development with potent pharmacological activities is needed to fight against CAUTI-causing organisms. Hence, our study investigated the methanolic extract of *Kalanchoe pinnata* (Lam.) Pers. for antimicrobial and antibiofilm activities against *Escherichia coli*, *Staphylococcus aureus*, *Candida albicans*, and *Enterococcus faecalis*, and the antimicrobial activity was proved by the agar diffusion method, and the least inhibitory concentrations of *K. pinnata* were found to be 0.5 mg/ml, which desired to inhibit *E. coli*, *S. aureus*, *C. albicans*, and *E. faecalis* growth. Further, *K. pinnata* extracts antibiofilm activity was quantified through the crystal violet staining method and efficiently inhibited all the test pathogens' biofilm formation on non-living surfaces up to their MIC concentrations. Also, the extract efficiently eradicated 83%, 86%, 89%, and 86% of *S. aureus*, *E. faecalis*, *E. coli*, and *C. albicans* mature biofilms, respectively, of all the test pathogens after 3X MIC (1.5 mg/ml) of *K. pinnata* extract. The catheter coating with *K. pinnata* extractrevealed antiadhesive properties against all the tested pathogens. In addition, the *K. pinnata* extract exhibited antioxidant properties and was not cytotoxic to normal L_{929} cells. In conclusion, based on the results, the *K. pinnata* extract may act as an alternative agent for CAUTI treatment.

1. Introduction

For several decades, implantable medical devices used for various reasons in the human body have gained much attraction in relation to biofilm-associated infection among hospitalised patients (Papanikolopoulou *et al*., 2022). Generally, device-related biofilm infections are initiated through colonisation on any living and non-living surfaces, such as artificial heart valves, cardiac pacemakers, urinary catheters, central venous catheters, needleless connectors, *etc*., which carry infections that were extremely affecting the patient's life, resulting in increased morbidity and mortality rates (Percival *et al*., 2015). Though several medical devices have been used, the urinary catheter significantly gained huge attention owing to its usage in healthcare support during patient hospital stays. More importantly, the catheter permits microbial entry to the sterile urinary system and makes it vulnerable for microbial colonisation, resulting in catheter-associated urinary tract infection (CAUTI), which denoted 60-70% of all nosocomial infections (Medina and Castillo-Pino, 2019; Skelton-Dudley *et al.,* 2019). During colonisation, the catheter provides enough surface for microbial attachment, which makes treatment difficult, resulting in a long hospital stay (Milo *et al*., 2019; Haque *et al*., 2018; Hollenbeak *et al*., 2018). Many reports indicated the microbes such as *S. aureus, E. faecalis, E. coli,* and *C. albicans* involved in CAUTI, which are biofilm-forming organisms (Sharma *et al*., 2016; Veerachamy *et al.,* 2014). The CAUTI-causing organisms can form a three-dimensional, well-organised, complex structure by attaching to the catheter surface and also produce extracellular polymeric substance (Bjarnsholt *et al*., 2016). These biofilms act as a barrier for microbes' protection from numerous environmental conditions, including chemical agents, pH, stress, *etc*., which makes biofilms harder for antibiotic treatment (Karigoudar *et al*., 2019) and leads to antibiotic resistance; thus infection related to biofilm has become more critical for elimination on catheter surfaces (Kurmoo *et al*., 2020; Sandhu *et al*., 2018). Hence, limited efficacy and higher resistance have determined the continuous search for new antimicrobials to decrease the microbial attachment as well as colonisation on the catheter surface.

In this scenario, the plant-based biomolecules are focused on several pharmaceutical interests, including antimicrobials, anticancer, antioxidants, anti-inflammatories, *etc.* (Sivakumar *et al*., 2022; Sri Bhuvaneswari *et al*., 2023; Karthikeyan *et al*., 2024). Based on this evidence, plants have been studied for various biomolecules against disease-causing pathogens. Hence, *K. pinnata*, a juicy plant that belongs to the Crassulaceae family and is widely used in traditional medicine for its various biological properties, including anticancer, antioxidant, and anti-inflammatory (Anandan *et al.,* 2024; Nascimento *et al*., 2023; Kolodziejczyk-Czepas *et al*., 2017). In addition, it was occupied as ethnomedicine for treating different conditions like insect bites, gastric ulcers, burns, abscesses, and peptic ulcers. Therefore, the present work investigated the antimicrobial and antibiofilm activities of *K. pinnata* against CAUTI-causing pathogens.

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2. Materials and Methods

2.1 Collection and authentication of the plant material

The plant material used in the present study was collected from a local nursery and was identified as *K. pinnata* (Lam.) Pers. by Dr. Mamoon AlFakhi, Chief Scientist, DM Institute of Biological Research, Omdurman, Sudan, with Authentication Number DMIBR/PA/03/2024.

2.2 Inoculum preparation

All the test pathogens, such as *E. coli, S. aureus, C. albicans,* and *E. faecalis* (0.5 Mac Farland unit), were used and grown in Brain Heart Infusion (BHI) broth. Whereas, *E. coli* and *C. albicans* were grown in Mueller Hinton broth (MHB) and Sabouraud Dextrose Broth (SDB). The positive controls, such as ampicillin, rifampicin, nystatin, and vehicle control methanol, were used for the study.

2.3 *K. pinnata* methanolic crude extract preparation

The *K. pinnata* powder purchased from the local market was weighed for 20 g and shifted to a cellulose thimble and placed inside the Soxhlet apparatus as dictated before (Harley *et al.,* 2022). The reactions were getting started once methanol was added and continued for several hours for a clear solution in the apparatus. The collocated final product was used as a crude extract.

2.4 *K. pinnata* methanolic crude extract antimicrobial activity

To determine the *K. pinnata* methanolic crude extractantimicrobial activity against *E. coli, S. aureus, C. albicans,* and *E. faecalis,* the well diffusion method was adapted (Meiyazhagan *et al*., 2016; Arghya Mani and Surajit Mitra, 2021). The plates prepared were swabbed with test cultures and received two different concentrations (1 mg/well and 2 mg/well) of *K. pinnata* methanolic crude extract and were allowed for zone formation.

2.5 *K. pinnata* methanolic crude extract MIC determination

The *K. pinnata* methanolic crude extractMIC was calculated against*E. coli, S. aureus, C. albicans,* and *E.faecalis* usingthe microdilution method as completed before (Meiyazhagan *et al*., 2015). The serial dilution of 4 mg/ml *K. pinnata* methanolic crude extract was performed to get 0.031 mg/ml of extract. Each was receiving overnight test cultures and incubated. Then, the optical density (OD) of each well was measured using a spectrophotometer (Apel PD 3000UV) at 600 nm.

2.6 *K. pinnata* methanolic crude extract effect on biofilm formation

The *K. pinnata* methanolic crude extract effecton *E. coli, S. aureus, C. albicans,* and *E. faecalis* biofilm formations was studied by the crystal violet method as presented previously (Meiyazhagan *et al*., 2015). In the presence of a varied range (4 mg to 0.031 mg/ml) of *K. pinnata* methanolic crude extract in the respective broth, it was allowed for 5 days and methanol fixation for attached cells followed by crystal violet staining. The final purple colour product was measured using a spectrophotometer (Apel PD 3000UV) at 570 nm after adding an ethanol-acetone mixture.

2.7 *K. pinnata* methanolic crude extract effect on mature biofilm eradication

The *K. pinnata* methanolic crude extract effecton mature biofilms of *E. coli, S. aureus, C. albicans,* and *E. faecalis* was quantified by the crystal violet method as displayed before (Meiyazhagan *et al.,* 2015). The *K. pinnata* methanolic crude extract 1X, 2X, and 3X MIC concentrations were used to treat 5-day mature biofilms of test pathogens followed by methanol fixation after treatment. The fixed cells stained with crystal violet and were detained with an ethanol and acetone mixture. The final product was read using a spectrophotometer (Apel PD 3000UV) at 570 nm.

2.8 *K. pinnata* methanolic crude extract coated catheter antimicrobial activity

A commercially purchased *in vitro* bladder was used to study theantimicrobial activity of *K. pinnata* methanolic crude extract-coated catheter tubes against *E. coli, S. aureus, C. albicans,* and *E. faecalis* (Goda *et al.* 2022). In brief, sterile Petri plates swabbed with test pathogens were placed by catheter tubes, which were coated by *K. pinnata* methanolic crude extractby soaking in it. The tubes were placed over the plates and incubated for growth inhibition.

2.9 Antioxidant property of *K. pinnata* methanolic crude extract

The *K. pinnata* methanolic crude extractantioxidant property was evaluated through DPPH (2, 2-diphenyl-1-picryhydrazyl) free radical scavenging assay as demonstrated before (Gayathri and Sathish Kumar, 2016). Briefly, the various *K. pinnata* methanolic crude extractconcentrations (0.5 mg/ml, 1mg/ml, 2 mg/ml, 3 mg/ml, 4 mg/ml, and 5 mg/ml) were permitted to react with DPPH solution for 30 min. The percentage of radical scavenging activity was calculated after measuring the final productusinga spectrophotometer (Apel PD 3000UV) at 517 nm.

2.10 *K. pinnata* methanolic crude extract cytotoxicity

The *K. pinnata* methanolic crude extractcytotoxicity was analysed on L_{929} (mouse fibroblast cells) through MTT assay as chosen earlier (Meiyazhagan *et al*., 2015). Shortly, *K. pinnata* methanolic crude extract at various concentrations (0.5 mg/ml, 1 mg/ml, 2 mg/ml, 3 mg/ml, and 4 mg/ml) was added to the DMEM (Dulbecco's Modified Eagles Medium), which contains cells, for 24 h, followed by formazan product formation by MTT addition. The percentage of cell viability was calculated after measuring the final product using a spectrophotometer (Apel PD 3000UV) at 570 nm.

2.11 Statistical analysis

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

3. Results

3.1 *K. pinnata* methanolic crude extract antimicrobial activity

K. pinnata methanolic crude extract antimicrobial activitywas investigated against *E. coli, S. aureus, C. albicans,* and *E. faecalis,* and the attained result is displayed in Figure 1. As noticed in the figure, *K. pinnata* methanolic crude extract at two concentrations showed activity against tested pathogens in the form of zone formation around the well, and the zone size increased when concentration increased, indicating activity is based on dose dependence.

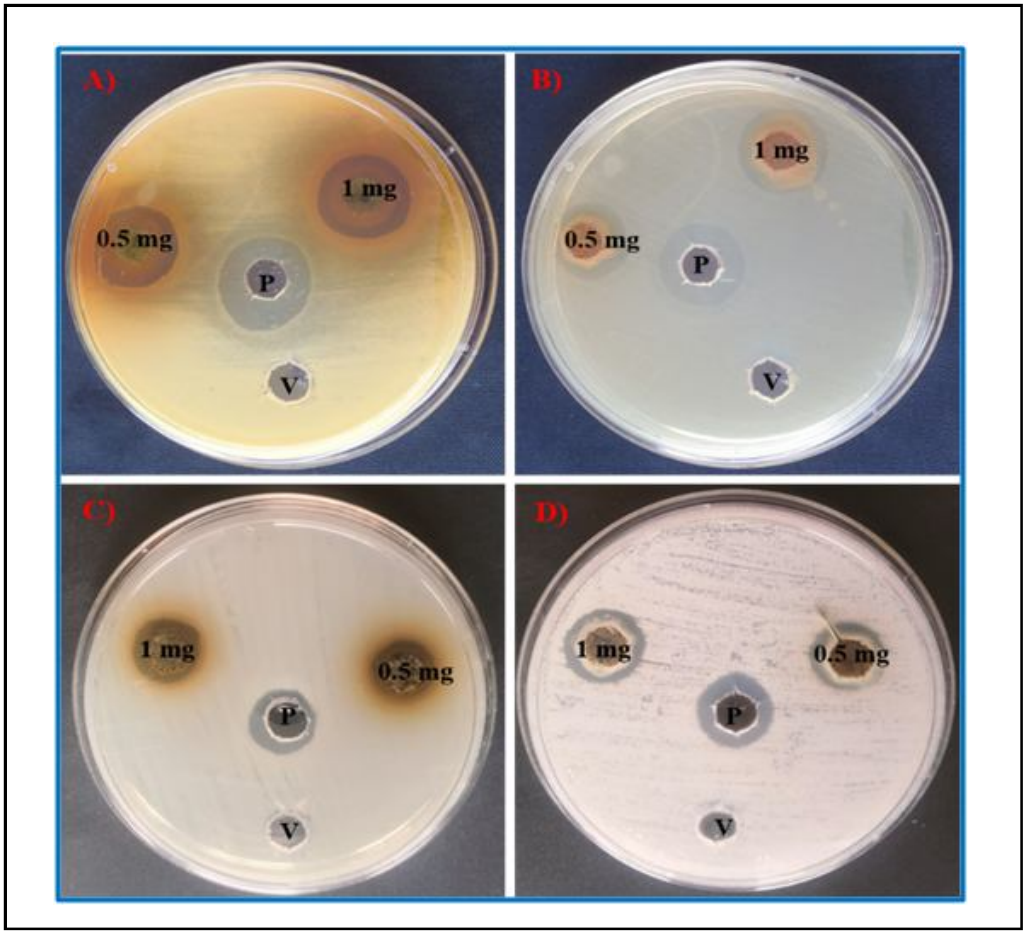

Figure 1: Antimicrobial activity of *K. pinnata* methanolic crude extract.
Note: P-positive controls (in the centre) and V-vehicle control. (A) *S. aureus*, (B) *E. faecalis*, (C) *E. coli*, and (D) *C. albicans.*

3.2 *K. pinnata* methanolic crude extract MIC determination

The *K. pinnata* methanolic crude extract MIC was determined against *S. aureus*, *E. faecalis, E. coli,* and *C. albicans,* and the minimal growth inhibitory concentrations of *K. pinnata* methanolic crude extract calculated are presented in Figure 2. As observed in the figure, 0.5 mg/ml of *K. pinnata* methanolic crude extract was desired to stop the growth of *E. coli, S. aureus, C. albicans,* and *E.faecalis.*

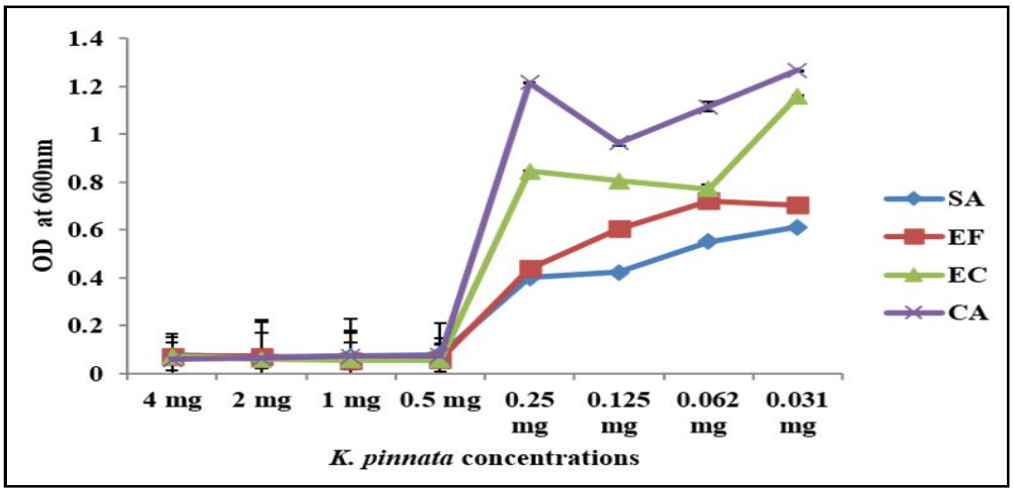

Figure 2: *K. pinnata* methanolic crude extract MIC determination.
Note: SA-*S. aureus*, EF- *E. faecalis*, EC- *E. coli*,and CA-*C. albicans*

3.3 *K. pinnata* methanolic crude extract effect on biofilm formation

The biofilm-forming abilityof *E. coli, S. aureus, C. albicans,* and *E. faecalis* in the occurrence of *K. pinnata* methanolic crude extract was estimated, and the biofilm formation quantified as a percentage after treatment with a wide range of concentrations (4 mg/ml to 0.031 mg/ml) is displayed in Figure 3. As noticed in the figure, the formation of biofilm was not evidenced up to their MIC, but a gradual increase of biofilm formation was noticed against all test pathogens after their MICs, which indicates the antibiofilm activity of *K. pinnata* methanolic crude extract.

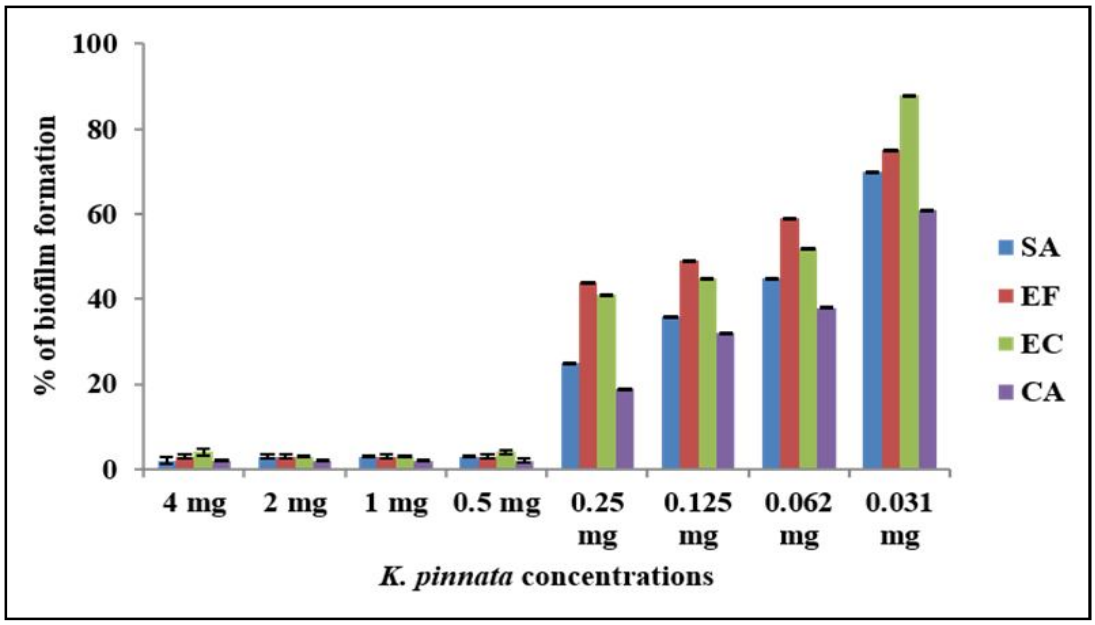

Figure 3: ***K. pinnata*** **methanolic crude extract effect on biofilm formation.**
Note: SA-*S. aureus*, EF-*E. faecalis*, EC-*E. coli*,and CA-*C. albicans*.

3.4 *K. pinnata* methanolic crude extract effect on mature biofilm eradication

The effect of *K. pinnata* methanolic crude extract on mature biofilms of *E. coli, S. aureus, C. albicans,* and *E. faecalis* quantified is presented in Figure 4. The figure represented the capacity of *K. pinnata* methanolic crude extract in eliminating test pathogens biofilm after three concentrations of treatment. The 1X, 2X, and 3X MIC concentrations of *K. pinnata* methanolic crude extract eradicated 73%, 83%, and 83% of *S. aureus* biofilms; 71%, 84%, and 86% of *E. faecalis* biofilm reduction; 73%, 79%, and 89% of *E. coli* biofilms; and 80%, 82%, and 86% of *C. albicans* biofilms, indicating *K. pinnata* methanolic crude extract's antibiofilm activity against test pathogens.

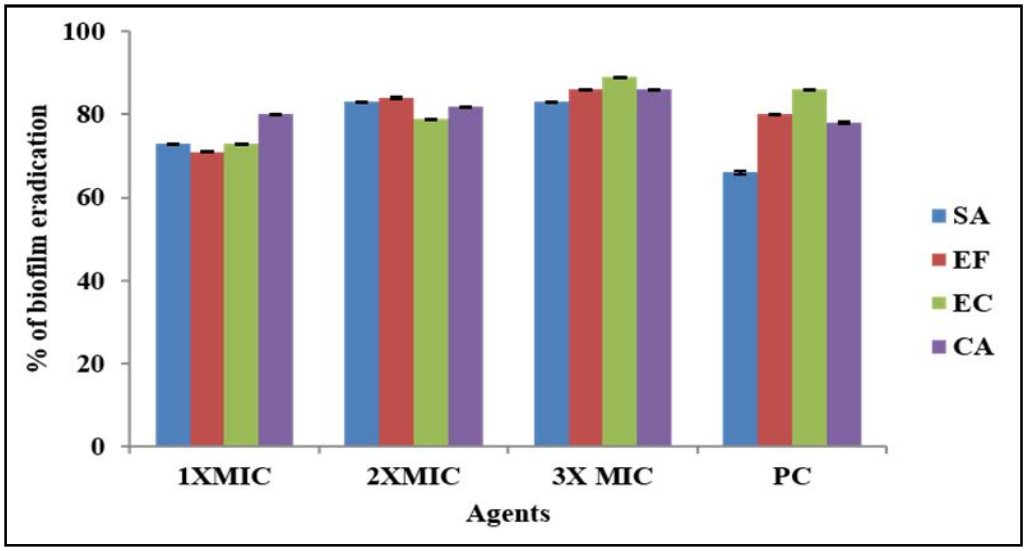

Figure 4: Quantitative biofilm eradication of *K. pinnata* methanolic crude extract.
Note: SA-*S. aureus*, EF-*E. faecalis*, EC-*E. coli*,and CA-*C. albicans*. PC-positive controls.

3.5 *K. pinnata* methanolic crude extract coated catheter antimicrobial activity

The silicone catheter tube coated with *K. pinnata* methanolic crude extract was studied for its antimicrobial activity against *E. coli, S. aureus, C. albicans,* and *E. faecalis* using an *in vitro* bladder model, and the observed zone of test pathogens inhibition is presented in Figure 5. The coated catheter tube showed zone formation surrounding the tube area when compared with the uncoated tube, where no zone was noted, and the antimicrobial activity of *K. pinnata* methanolic crude extract test pathogens was evidenced.

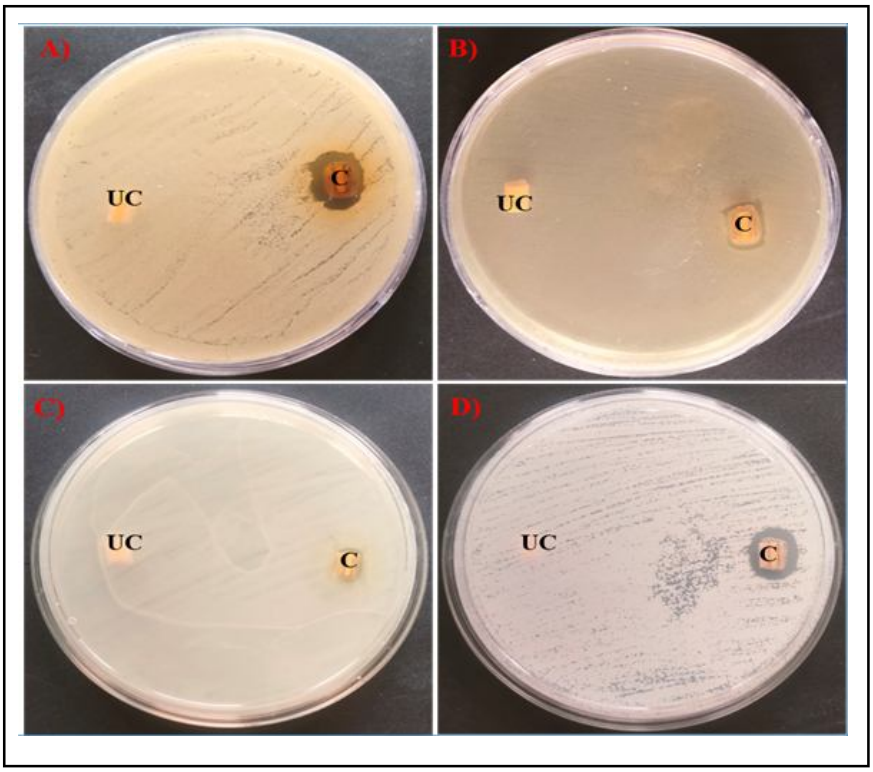

Figure 5: Antiadhesive property of *K. pinnata* methanolic crude extract coated catheter. (A) *S. aureus*, (B) *E. faecalis*, (C) *E. coli*, and (D) *C. albicans*.

Note: UC-uncoated, C-coated with *K. pinnata* methanolic crude extract.

3.6 *K. pinnata* methanolic crude extract antioxidant property

The antioxidant property of *K. pinnata* methanolic crude extract was investigated using DPPH, and the calculated free radical scavenging percentage after treatment is presented in Figure 6. The figure represented the wide range of *K. pinnata* methanolic crude extract (0.5 mg/ml, 1 mg/ml, 2 mg/ml, 3 mg/ml, 4 mg/ml, and 5 mg/ml), Showing 17%, 31%, 58%, 71%, 77%, and 84% scavenging activity was noted after treatment.

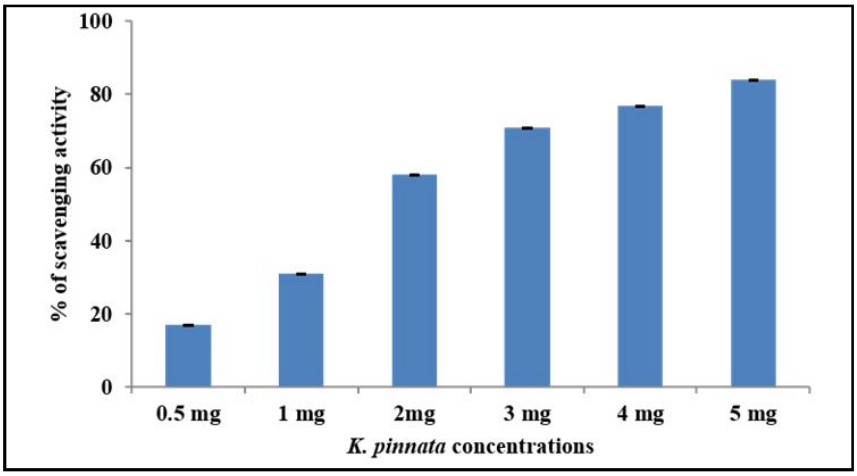

Figure 6: The antioxidant property of *K. pinnata* methanolic crude extract.

3.7 *K. pinnata* methanolic crude extract cytotoxicity

The *K. pinnata* methanolic crude extract cytotoxicity investigated on L_{929} cells using MTT is displayed in Figure 7. In the figure, cell viability percentage calculated after receiving various concentrations such as 0.5 mg/ml, 1 mg/ml, 2 mg/ml, 3 mg/ml, and 4 mg/ml of *K. pinnata* methanolic crude extract showed 93%, 91%, 73%, 61%, and 53% cell viability after treatment, indicating the MIC concentration was not cytotoxic to normal cells.

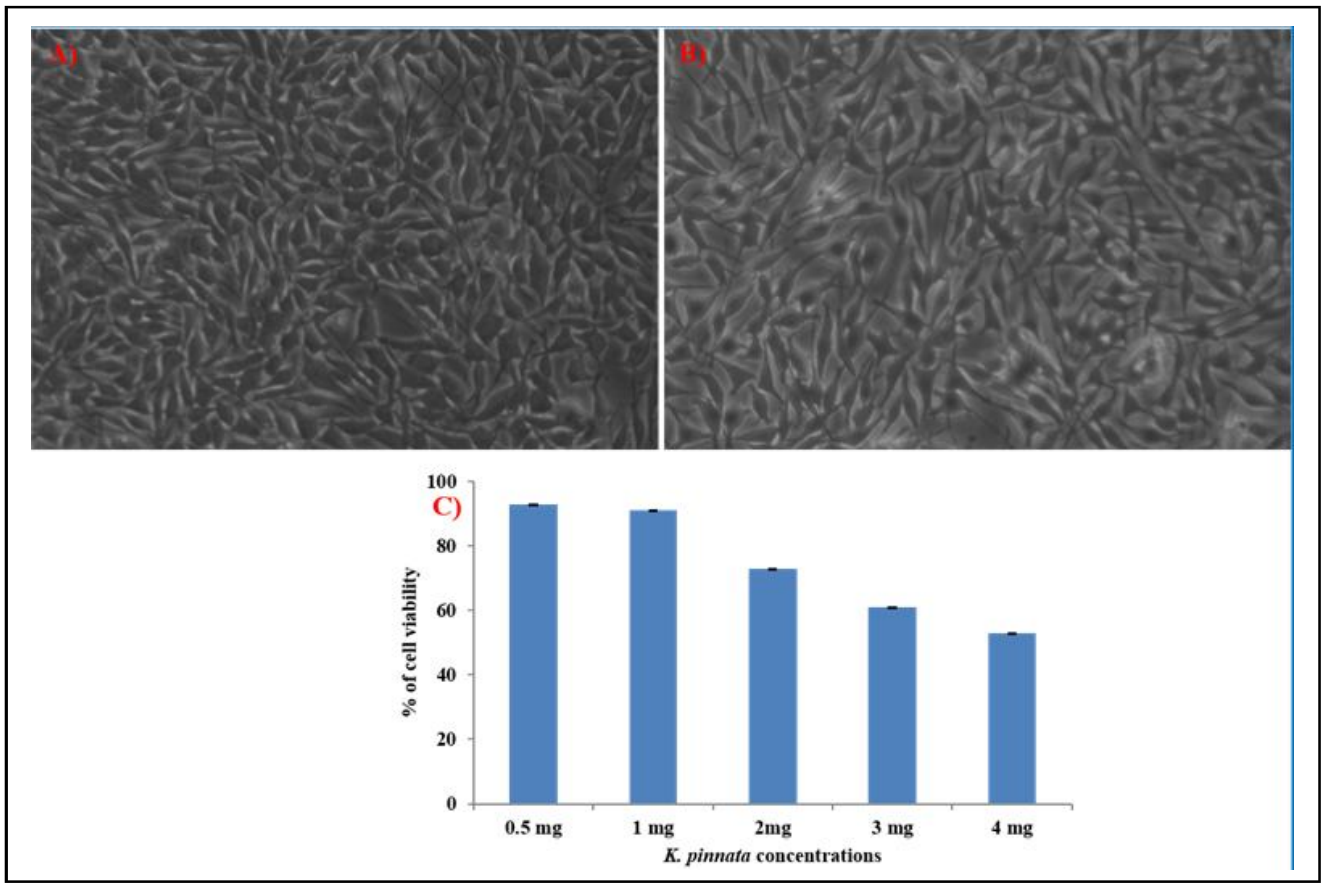

Figure 7: The *K. pinnata* methanolic crude extract cytotoxicity on L_{929} cells. (A) Untreated cells, (B) Treated with *K. pinnata* methanolic crude extract and (C) Graph represents cell viability percentage after treatment with *K. pinnata* methanolic crude extract various concentrations.

4. Discussion

CAUTI is one of the important biofilm-related infections that causes drug resistance, resulting in treatment failure in hospitalised patients. The long-term catheter usage promotes the biofilm formation, thereby making the treatment process critical, resulting in high morbidity and mortality. Therefore, the situation triggered for drug development with potential antimicrobial and antibiofilm activities is needed to fight against pathogens. In the present study, the authors were analysing the antimicrobial potential of one of the long-used medicinal plants. *K. pinnata*, using its methanolic extract, the four most important uropathogens, *viz., E. coli, S. aureus, C. albicans,* and *E. faecalis.* The study was supported by various groups, wherein the *K. pinnata* leaf juice established significant antibacterial activity against *E. coli*, *Staphylococcus* sp., *Bacillus* sp., *Shigella* sp., *P. aeruginosa,* and some multidrug-resistant bacteria (Joseph *et al*., 2011), and the methanolic extract of *K. pinnata* showed activity against the most important human pathogens like *S. dysenteriae, E. coli, P. vulgaris, B. subtilis,* and *S. aureus* (Akinpelu, 2000). A report says the active compounds, such as alkaloids and flavonoids, extracted from the ethanolic extract of *K. pinnata* showed antimicrobial activity, which efficiently prevented the growth of many Gram-positive and Gram-negative bacteria (Etim *et al*., 2016; Okwu and Nnamdi, 2011). Similarly, three bioactive compounds extracted from *K. pinnata* leaves were tested against respiratory infection-causing bacteria and showed a significant effect of antibacterial activity, which was correlated with the traditional method used for respiratory tract infections (Richwagen *et al*., 2019; Mudi and Ibrahim, 2010).

The *K. pinnata* antibiofilm activity was examined against test pathogens to test the ability to eliminate biofilms from non-living surfaces. The catheter lumen acts as an entrance for microbes during catheterisation and forms biofilm by adopting various survival steps, including attachment, colony formation, and maturation, which is embedded with a complex three-dimensional structure that makes treatment ineffective (Pelling *et al*., 2019; Zhu *et al*., 2019). Therefore, the study aimed to concentrate on each stage of biofilm formation, and *K. pinnata* had potential antibiofilm activity against test pathogens biofilms by inhibiting and eradicating five-day mature biofilms. Consequently, the catheter allowed bacterial colonisation on the inner

and outer surfaces, resulting in biofilm formation. To prevent bacterial colonisation, the coating of catheter surfaces with methanolic extract of *K. pinnata* is an excellent method to eradicate biofilm on catheter surfaces. Keeping this in mind, the antimicrobial activity of *K. pinnata* catheter coating against test pathogens was examined, and the activity was found by the clear zone round the tube. Likewise, the present study was supported by numerous studies wherein various antimicrobial agents, such as polymers, zinc oxide, some antibiotic combinations, silver, and fosfomycin, were coated on catheter surfaces and tested against *E. faecalis*, *E. coli*, *P.aeruginosa*, *K. pneumoniae,* and *C. albicans* (Aleksandra *et al*., 2021; Jia *et al*., 2021; Rahuman *et al*., 2021; Abbott *et al.,* 2020; Fisher *et al*., 2015). Overall, the findings established the usefulness of *K. pinnata* extract as a promising antimicrobial agent for the treatment of CAUTI infection.

5. Conclusion

The *K. pinnata* methanolic extract showed antimicrobial activity with the least inhibitory concentrations against tested pathogens. The test pathogens biofilm inhibition and eradication of mature biofilms were noticed after *K. pinnata* treatment. The catheter coating with *K. pinnata* extract showed antimicrobial activity in an *in vitro* bladder model that mocked the suitable environment. Overall, *K. pinnata* may bean alternative agent for CAUTI infection.

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Conflict of interest

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

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