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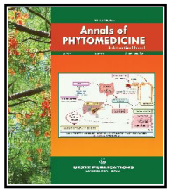

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# Antibacterial and antibiofilm activities of methanolic extract of *Indigofera tinctoria* L. against catheter-associated urinary tract infection (CAUTI) causing pathogens

**Gobianand Kuppannan*, K.P. Shamna** and Muhammad Musthafa Poyil*****◆

**Department of Microbiology, Noorul Islam College of Dental Sciences, Aralumoodu, Athiyanoor, Thiruvananthapuram-695123. Kerala, India*
*** Department of Obstetrics and Gynaecology, Faculty of Medicine in Al-Qunphudah, Umm Al-Qura University, Saudi Arabia.*
**** Department of Basic Medical Sciences, College of Medicine, Prince Sattam bin Abdulaziz University, Al-Kharj 11942, Saudi Arabia.*

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**Abstract**

Microbial attachment on the surface of the catheters during the insertion of the catheter leads to catheter-associated urinary tract infection (CAUTI) which denotes a very important nosocomial infection mainly associated with the formation of biofilms by the causative microorganisms, resulting in serious socio-economic implications due to treatment challenges leading to higher morbidities and increased mortalities. As these pathogens are resistant to many of the antibiotic drugs available, the scientific community is investigating new antibiotics of natural origin and the phytocompounds gain much attraction owing to their pharmacological benefits. So, in a trial to contribute to this investigation, the present study analyzed the methanolic extract of *Indigofera tinctoria* L. for its antibacterial, antibiofilm, and antiadhesive properties against *Staphylococcus aureus*, *Candida albicans*, *Escherichia coli,* and *Enterococcus faecalis* which are among the mainly listed organisms causing CAUTI. The well-diffusion method confirmed the potentials of *I. tinctoria* extract against *S. aureus*, *C. albicans*, *E. coli*, and *E. faecalis*, and the minimal inhibitory concentrations (MICs) of *I. tinctoria* were found to be 0.312 mg/ml against each of them except for *S. aureus* for which it was 0.156 mg/ml). Moreover, *I. tinctoria* was also studied for its effect on biofilm formation, and biofilm eradication, and it was found that the extract could inhibit the biofilms at its MIC level. Additionally, *I. tinctoria* treated with 1X MIC of the extract effectively eradicated the biofilms by the microorganisms, *E. coli, C. albicans, S. aureus,* and *E. faecalis* by 76%, 77%, 84%, and 71%, respectively. Further, *I. tinctoria* coated catheter tube showed antibacterial activity against test organisms. The extract also showed promising antioxidant properties when analyzed using DPPH assay Further, the analyses for cytotoxicity on $L_{929}$ cells proved that the extract at that particular concentration did not possess any toxic effects on the eukaryotic cells. Thus, the study recommends further *in vivo* investigations with purified phytocompounds to explore the possibilities for *I. tinctoria* extracts to be used in the treatment of the CAUTIs caused by the mentioned pathogens.

## 1. Introduction

Implantable medical devices have become one of the most important parts of the treatment in clinical settings which provide support and healthcare quality maintenance in response to the increase in disease prevalence. Nevertheless, the exterior implantation in the body is frequently escorted by various nosocomial infections (Papani-kolopoulou *et al*., 2022). More importantly, 60-70% of nosocomial infections are mainly related to implants or biomaterials which have an enormous impact on the healthcare system by prolonged hospital stay, increased medical costs, *etc.* (Skelton-Dudley *et al.,* 2019; Medina and Castillo-Pino, 2019; Yasir *et al*., 2018). Implantable medical device-associated biofilm infections are mostly caused by microbial colonization on non-living devices including urinary catheters, artificial heart valves, needleless connectors, cardiac pacemakers, central venous catheters, *etc*., and carry the risk of biofilm-associated infections which tremendously affect the quality of life and even threaten the life resulting high rate of mortality and morbidity (Damyanova *et al.,* 2024; Percival *et al*., 2015). Catheter-associated urinary tract infections (CAUTIs) are more prevalent among several implant-related infections, and the biofilm-forming microorganisms in the inner surfaces of catheters with long-term usage can resist antibiotic treatment resulting in chronic infections (Delcaru *et al*., 2016; Leo *et al*., 2014). Several studies reported the causative agents responsible for CAUTIs and they include the most prevalent ones like *Staphylococcus aureus, Candida albicans, Escherichia coli,* and *Enterococcus faecalis,* and they are acquired from endogenous microbiota of the perineum that rise to the urethra and bladder along the catheter's external surface (Carvalho *et al*., 2020; Mishra *et al.,* 2020; Sharma *et al*., 2016; Veerachamy *et al.,* 2014). These CAUTI-causing microbes are biofilm-forming and they adhere to the surface of medical devices making the organisms more resistant to the existing antibiotics resulting in antimicrobial resistance (Bjarnsholt *et al*., 2016).

**Corresponding author: Dr. Muhammad Musthafa Poyil**
*Department of Basic Medical Sciences, College of Medicine, Prince Sattam bin Abdulaziz University, Al-Kharj-11942, Saudi Arabia.*
**E-mail: m.poyil@psau.edu.sa**
**Tel.: +966-565634412**

Biofilms are three-dimensional well well-organized complex structures that have self-produced extracellular polymeric substances that are able to attach to the catheter substances (Sharma *et al*., 2023; Pallee *et al*., 2023; Sharma *et al.,* 2019; Karygianni *et al.,* 2020). The biofilm matrix behaves as a barrier to protect the microorganisms from various environmental conditions such as stress, chemical agents, pH, *etc*., leading to multi-species populations with altered phenotypes to make the biofilms stronger and to escape the individual microorganisms from the treatment of antibiotics (Bano *et al*., 2023; Mirghani *et al*., 2022*;* Yin *et al.,* 2019; Karigoudar *et al*., 2019). Hence, biofilm development has been importantly raised and described as one of the resistant mechanisms, and thereby biofilm-associated infections have become more crucial to eliminate on the catheter surfaces (Asma *et al*., 2022; Irene *et* al., 2021; Walker *et al.,* 2020; Balcazar *et al*., 2015). The increased resistance and limited efficacy of antibiotics as well as alternative conventional methods have driven the search for novel effective replacements for preventing the adhesion of the microorganisms on the catheter's surface. As the plants have a variety of potential antibacterial biomolecules against pathogenic bacteria (Pragyandip *et al.,* 2015; Sethi and Singh, 2015), the present study selected the plant *I. tinctoria* for its potential against CAUTI-causing organisms. *I. tinctoria* is a type of shrub used traditionally in Indian, African, and Chinese medicine for various treatments including heart palpitation, constipation, liver diseases, cancer, *etc*., and also as food colorant, phytotoxic, insecticide and antiulcerogenic (Rakwa *et al.,* 2022; Pushpangadan *et al*., 2015; Ramanibai *et al*., 2014; Renukadevi and Sultana, 2011). The plant has shown the presence of various biochemical compounds like reducing sugars, alkaloids, glycosides, phenolic compounds, terpenoids, flavonoids, saponins, carbohydrates, *etc.* (Chandrasekaran, 2018; Verma and Suresh, 2002). Even though, there are some studies conducted using the leaves of *I. tinctoria*, which displayed antibacterial potentials against Gram-positive pathogens, the study reports are limited on the potentials of the plant against medically important pathogens. Thus, the study investigated the antibacterial, antibiofilm activity, and antiadhesive properties of the methanolic extract of *I. tinctoria* against *S. aureus, C. albicans*, *E. coli,* and *E. faecalis* which are mainly listed as the causative agents in CAUTI biofilms.

## 2. Materials and Methods

### 2.1 Inoculum preparation

For all the assays, the overnight cultures of *S. aureus* (ATCC # 12600)*, C. albicans* (ATCC # 2091)*, E. coli* (ATCC # 13706)*,* and *E. faecalis* (ATCC # 14506) were procured form Microbiologique, USA and the cultures were adjusted to the standard level (0.5 MacFarland unit) and used. For the growth of *S. aureus* and *E. faecalis,* Brain Heart Infusion (BHI) broth, for *E. coli* Mueller-Hinton broth (MHB), and *C. albicans* Sabouraud's dextrose broth (SDB) were used. All the experiments were conducted in triplicate. Ampicillin, rifampicin, and nystatin were the positive controls and methanol was used as vehicle control.

### 2.2 *Indigofera tinctoria* methanolic crude extract preparation

For crude extract preparation, in a fresh cellulose thimble, 20 g of *I. tinctoria* leaf of the plant authenticated (No. DAP/MPA-23/2024) by Dr. Shamna, Technical Supervisor, Deseeya Ayurvedic Pharmacy, Kozhikode, Kerala, India was added and kept inside the Soxhlet apparatus as per the standard protocols (Harley *et al.,* 2022). The reaction was started when adding an adequate volume of methanol to the flask and the temperature was kept at 60$^{o}$C (being the boiling point is comparatively lower, methanol was selected as the solvent) for several hours to run the reaction cycles. The reaction was stopped when the colorless solvent was attained in the Soxhlet apparatus and solvent evaporation was performed to get the final product which was used further for all the studies.

### 2.3 *Indigofera tinctoria* methanolic crude extract antimicrobial activity

The antimicrobial activities of *I. tinctoria* methanolic crude extract were studied against *S. aureus, C. albicans, E. coli,* and *E. faecalis* through the well-diffusion method (Meiyazhagan *et al*., 2016). In brief, all the overnight cultures of the above-specified test organisms were swabbed on respective sterile petri plates, and then, different concentrations of *I. tinctoria* methanolic crude extracts were added into respective wells and incubated overnight. The zone of inhibition surrounding the well indicates the antimicrobial activities of *I. tinctoria* methanolic crude extract against *S. aureus, C. albicans, E. coli,* and *E. faecalis*.

### 2.4 *Indigofera tinctoria* methanolic crude extract MIC determination

*I. tinctoria* methanolic crude extract MIC was determined using the microdilution method against *S. aureus, C. albicans, E. coli,* and *E. faecalis* (Meiyazhagan *et al*., 2015). In short, 1.25 mg/ml of *I. tinctoria* methanolic crude extract was diluted serially until the final concentration reached 0.009 mg/ml in the respective broth and was subjected to incubation for overnight after adding the respective culture in the respective plate. The optical density (OD) was read at 600 nm after the incubation.

### 2.5 *Indigofera tinctoria* methanolic crude extract effect on biofilm formation

The effect of *I. tinctoria* methanolic crude extract on biofilm formation of *E. coli*, *S. aureus, C. albicans*, and *E. faecalis* was investigated using crystal violet assay (Meiyazhagan *et al*., 2015). The biofilm formation of test organisms was studied in various concentrations of *I. tinctoria* ranging from 1.25 mg/ml to 0.009 mg/ml and allowed for five days. Later, the biofilms in the wells were washed followed by methanol fixation. Then, the crystal violet staining was done for fixed cells followed by destaining with ethanol acetone mixture. The attained final purple product was read at 570 nm.

### 2.6 *Indigofera tinctoria* methanolic crude extract effect on mature biofilms

*I. tinctoria* methanolic crude extract's effect on mature biofilms of *S. aureus, C. albicans, E. coli*, and *E. faecalis* was studied using crystal violet assay (Meiyazhagan *et al*., 2015). All the test organisms were allowed for 5 days to form biofilms and washed biofilms were treated with three different concentrations (1X, 2X, and 3X MIC) of *I. tinctoria*. The treated as well as untreated biofilms were fixed followed by crystal violet staining. Then, the stained biofilms were destained with a mixture of ethanol acetone solution. The OD was attained at 570 nm for the final purple product.

### 2.7 Antibacterial activity of *I. tinctoria* methanolic crude extract coated catheter

The antibacterial activity of *I. tinctoria* methanolic crude extract coated catheter was evaluated against *S. aureus, C. albicans, E. coli,* and *E. faecalis* using an *in vitro* bladder model (Goda *et al.,* 2022).

The small pieces of sterile silicone catheter were dipped in the solution of *I. tinctoria* methanolic crude extract and air dried and repeated twice. The air-dried and antimicrobial agent-coated catheter pieces were placed over the surface of the respective plate which was swabbed with respective organisms followed by overnight incubation. The zone around the catheter piece indicated the antimicrobial activity of *I. tinctoria* methanolic crude extract against test organisms.

### 2.8 Antioxidant property of *I. tinctoria* methanolic crude extract

Using free radical scavenging assay (2,2-diphenyl-1-picryhydrazyl - DPPH), the antioxidant properties of *I. tinctoria* methanolic crude extract were evaluated (Gayathri and Sathish Kumar, 2016) using ascorbic acid as the standard drug. Different extract concentrations (2.5 1.25, 0.625, 0.032, and 0.15 mg/ml) were mixed with 0.1 mM DPPH solution for 30 min. The OD was read at 517 nm for the final product and the radical scavenging activity percentage of *I. tinctoria* was calculated as shown:

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

### 2.9 Cytotoxicity of *I. tinctoria* methanolic crude extract

The cytotoxicity of *I. tinctoria* methanolic crude extract was investigated on $L_{929}$ (mouse fibroblast cell line) using an MTT assay (Meiyazhagan *et al*., 2015). Dulbecco's Modified Eagles Medium (DMEM) with 10% fetal bovine serum was used to grow the cells and they were treated with various concentrations (2.5 1.25, 0.625, 0.032, and 0.15 mg/ml) of *I. tinctoria* and incubated. Then, the formazan product was formed by adding 20 µl of MTT solution (5 mg/ml in PBS) followed by incubation for 3 to 4 h at 37°C. OD was measured at 570 nm and 690 nm after the formazan crystals were dissolved by adding DMSO. The cell viability percentage after treatment with *I. tinctoria* was calculated using the formula:

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 standard error.

## 3. Results

### 3.1 Antibacterial activity of *I. tinctoria*

The *I. tinctoria* antibacterial activities of the plant extract against *S. aureus, C. albicans, E. coli,* and *E. faecalis* were studied using the well-diffusion protocols, and the obtained result is displayed in Figure 1. Various concentrations of *I. tinctoria* methanolic crude extract showed a zone of inhibition around the well. The inhibition zone size was increased during the concentration increase indicating the activity is concentration-dependent against *S. aureus, C. albicans, E. coli,* and *E. faecalis*.

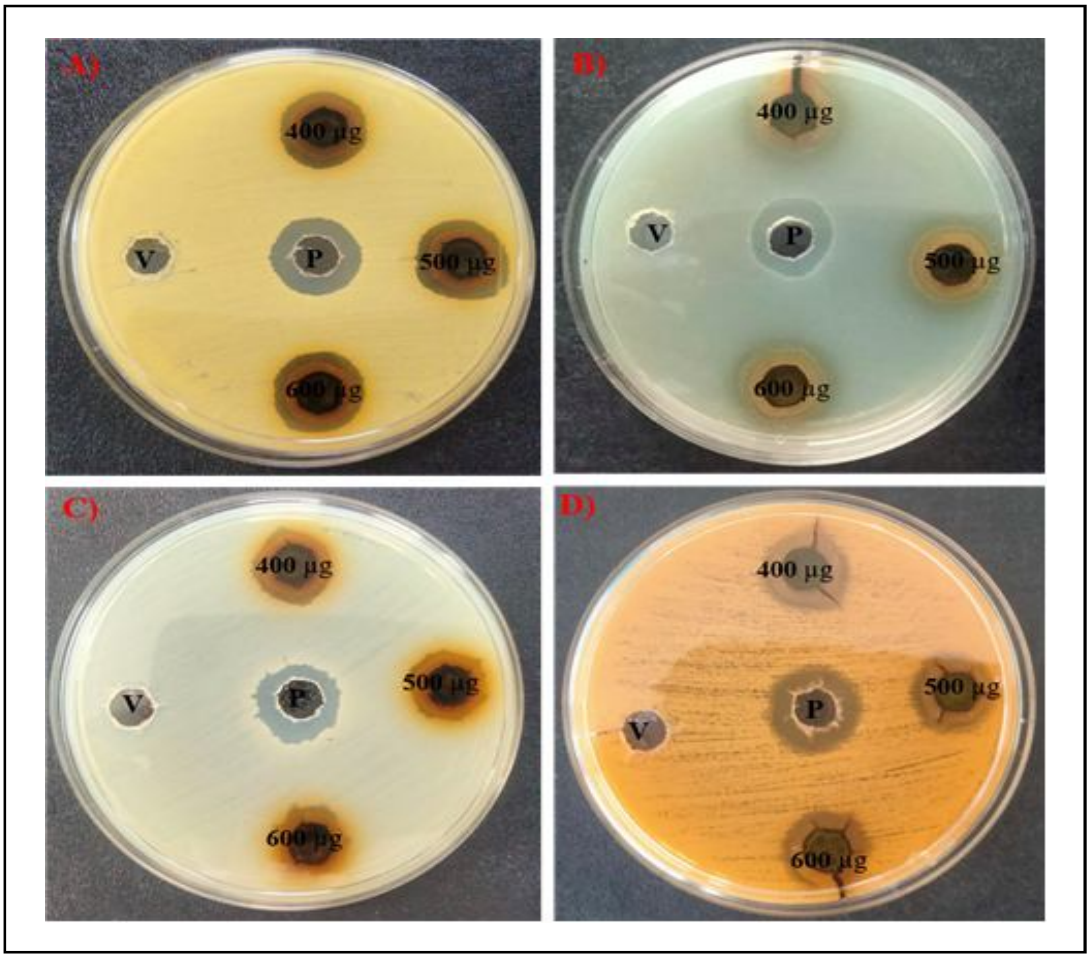


**Figure 1: Antibacterial activity of *I. tinctoria* against: A. *S. aureus,* B. *E. faecalis* C. *E. coli* and D. *C. albicans*.**

### 3.2 Minimal inhibitory concentrations MIC determination of *I. tinctoria*

The MICs *I. tinctoria* calculated against *S. aureus, C. albicans, E. coli,* and *E. faecalis* using the microdilution method are presented in Figure 2. As seen in the graph, 0.312 mg/ml was the MIC against all the tested microorganisms except for *S. aureus*, for which it was 0.156 mg/ml.

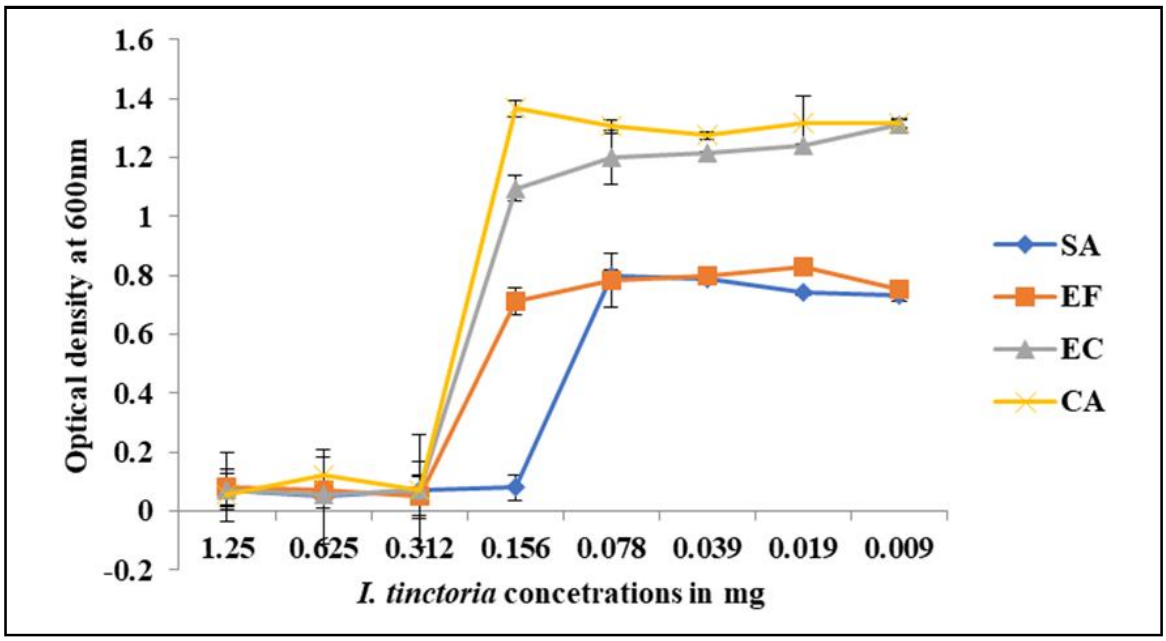


**Figure 2: MIC determination of *I. tinctoria* against *S. aureus, E. faecalis, E. coli,* and *C. albicans.***

### 3.3 *I. tinctoria* methanolic crude extract effect on biofilm formation

*I. tinctoria* methanolic crude extract was investigated for its potential to prevent the formation of biofilms by *S. aureus, C. albicans, E. coli,* and *E. faecalis* on polystyrene surface, and the calculated percentage of biofilm formation after treatment with various concentrations of *I. tinctoria* is presented in Figure 3. As observed in Figure, the *I. tinctoria* methanolic crude extract effectively inhibited the biofilm till its MIC level against all the tested organisms. A gradual increase in the biofilm formation was noticed below the MIC levels.

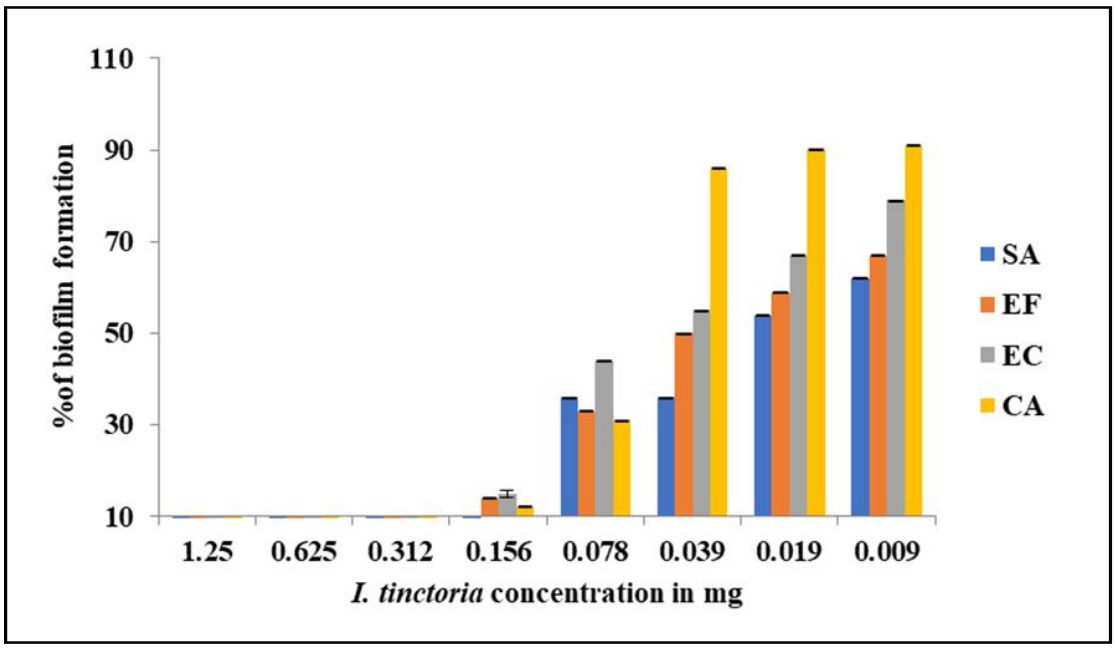


**Figure 3: Effect of *I. tinctoria* on biofilm formation of *S. aureus, E. faecalis, E. coli,* and *C. albicans.***

### 3.4 Effect of *I. tinctoria* methanolic crude extract on mature biofilms

*I. tinctoria* methanolic crude extract was tested for its effect on *S. aureus, C. albicans, E. coli,* and *E. faecalis* mature biofilms, and the obtained biofilm eradication percentage is presented in Figure 4. 1X MIC, 2X MIC, and 3X MIC of *I. tinctoria* treatment effectively eradicated biofilms by the test organisms. The increased biofilm eradication was noted in the highest concentration of *I. tinctoria* against testing each of the organisms.

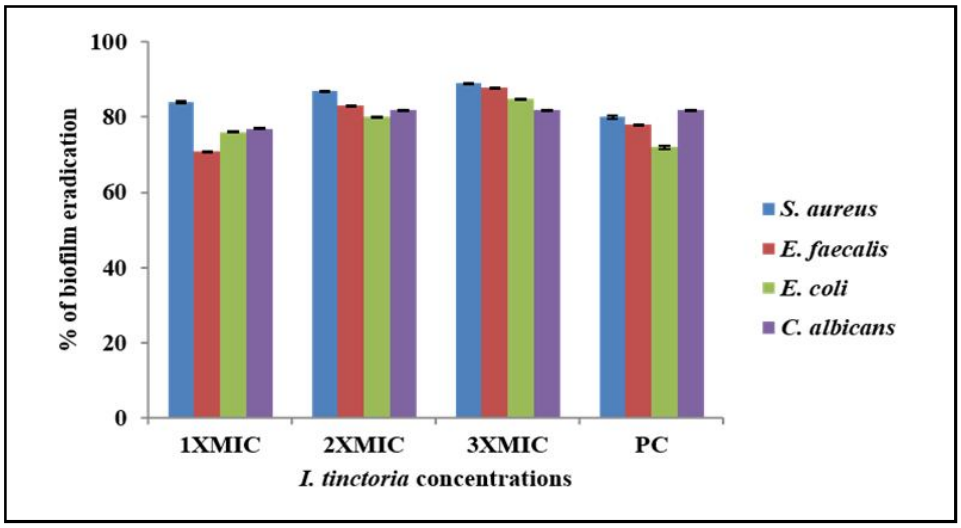


**Figure 4:** ***I. tinctoria*** **effect on biofilm eradication of** ***S. aureus, E. faecalis, E. coli,*** **and** ***C. albicans.***

### 3.5 Antibacterial activity of *I. tinctoria*-coated catheter

The methanolic extract-coated catheter tube was evaluated for its antimicrobial potential against *C. albicans, E. coli, S. aureus,* and *E. faecalis,* and the zone of inhibition around the drug-coated tube is displayed in Figure 5. The clear zones indicated the antimicrobial as well as antiadhesive properties of *I. tinctoria* against test organisms.

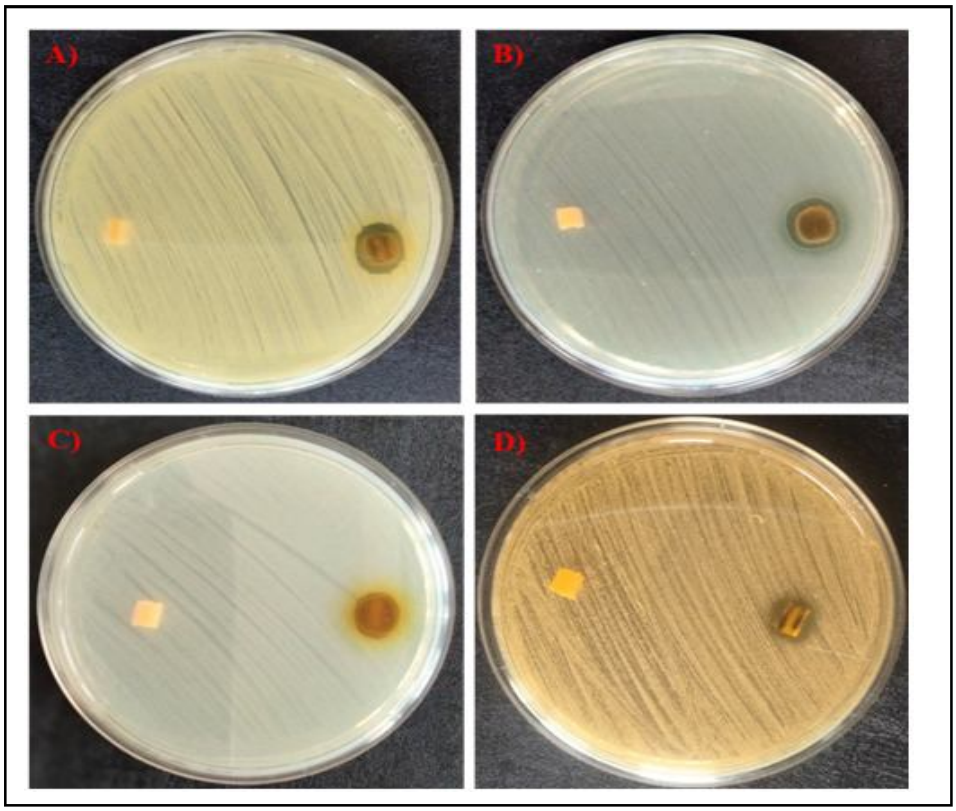


**Figure 5: Antibacterial activity of** ***I. tinctoria*** **methanolic extract coated catheter against: A.** ***S. aureus,*** **B** ***E. faecalis,*** **C.** ***E. coli*** **and D.** ***C. albicans.***

### 3.6 Antioxidant properties of *I. tinctoria*

The scavenging activity of *I. tinctoria* was determined and the calculated inhibitory effect is presented in Figure 6. As seen in the graph, the inhibitory percentage of *I. tinctoria* after treatment with various concentrations (2.5 1.25, 0.625, 0.032, and 0.15 mg/ml) was calculated as 86% at 2.5 mg of the extract.

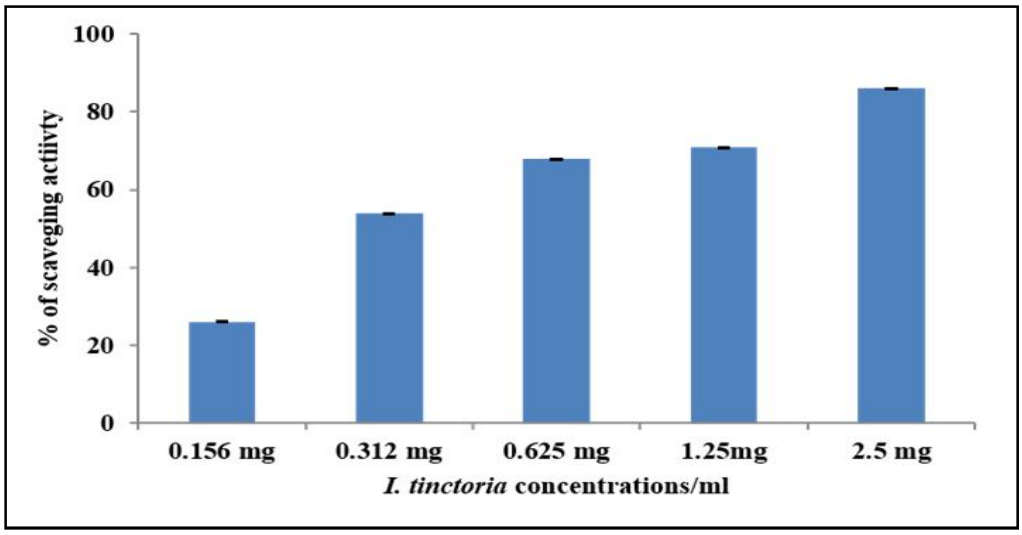


**Figure 6: Antioxidant property of *I. tinctoria* methanolic crude extract.**

### 3.7 Cytotoxicity

The methanolic crude extract *I. tinctoria* was investigated for its cytotoxic effect on $L_{929}$ using MTT assay and the calculated cell viability after treatment with various concentrations (2.5 1.25, 0.625, 0.032, and 0.15 mg/ml) is presented in Figure 7. As noted in Figure, the extract showed no cytotoxicity on the normal mice fibroblast cells.

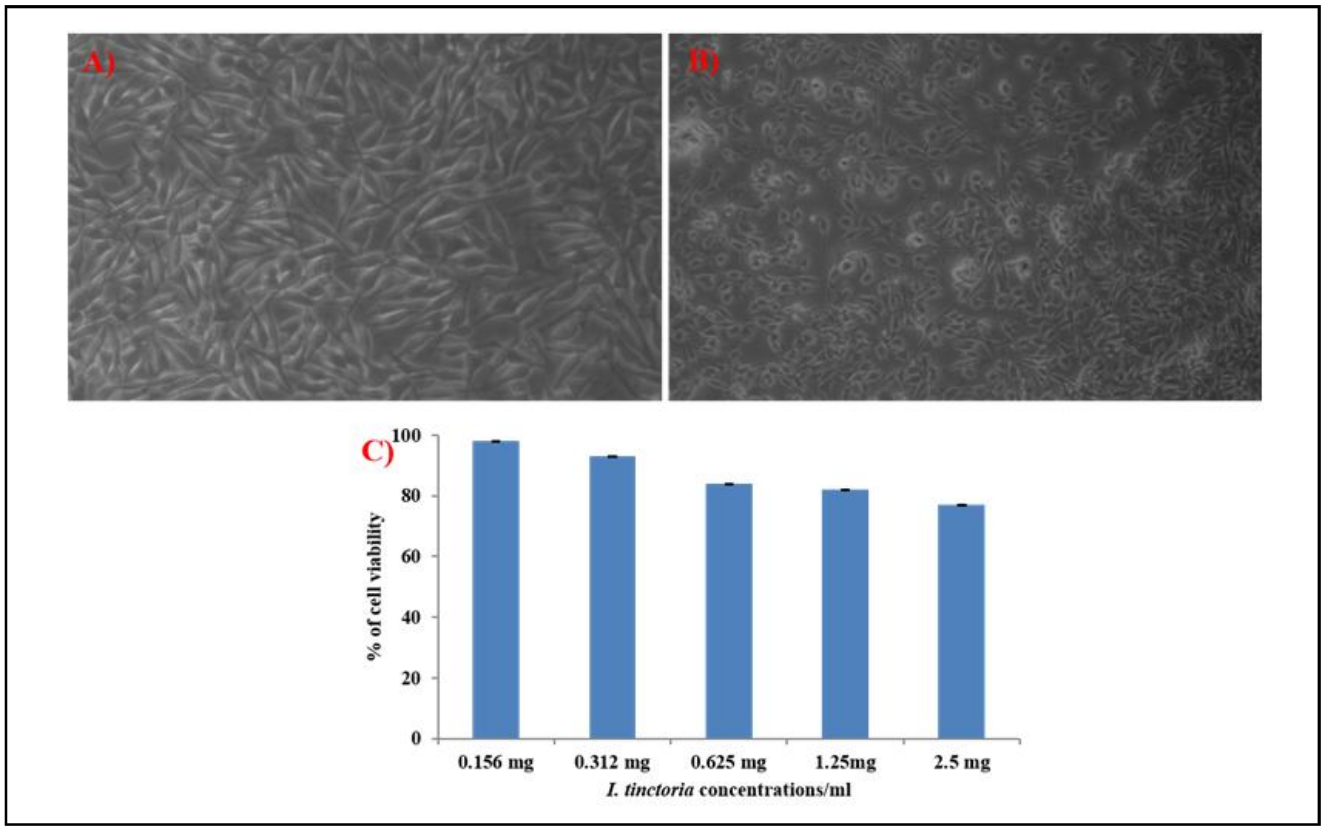


**Figure 7: Cytotoxic effect of *I. tinctoria* towards $L_{929}$ cells.**

## 4. Discussion

Catheter-associated urinary tract infection (CAUTI) is gaining much attention in healthcare settings owing to its serious complications in hospitalized patients. The biofilm-related CAUTIs make treatment challenges resulting in antibiotic-resistant strain development followed by management very tough. Therefore, our study investigated the methanolic extract of *I. tinctoria* against *S. aureus, C. albicans, E. coli,* and *E. faecalis* are frequently isolated organisms. Our study proved the antimicrobial activities of the plant *Indigofera tinctoria* against the above-mentioned microorganisms and found the least inhibitory concentration against all the tested organisms. In support of this, a recent study reported the ethanolic crude extract of *I. tinctoria* was evaluated against *S. epidermis* and *S. aureus* and also, found the least inhibitory concentrations (Dagu *et al*., 2021). Another study (Talha *et al.,* 2022) used this plant material as a reducing agent for the green synthesis of silver nanoparticles, and it was evaluated for its antibacterial activity against many microbes, and the highest activity was found against *E. coli*. However, our study reported that the highest activity was observed for *S. aureus*. Another study evaluated *I. tinctoria* for its antibacterial effect on various *S. aureus* sp. The study determined the MICs as *S. aureus* (0.125 µg/ml), vancomycin-resistant *Enterococcus* species (0.125-0.5 µg/ml), *Haemophilus influenzae* (2-4 µg/ml), coagulase-negative *S. aureus* (0.25-0.5 µg/ml) and *Moraxella* sp. (2 µg/ml). The highest MIC value recorded was against *S. aureus* and it could be due to strain variations (Mahesh *et al*., 2012). Another study revealed the *I. tinctoria* phytochemical analysis and the methanolic leaf and root extracts tested against *S. aureus, Klebsiella pneumoniae, E. faecalis, Enterobacter aerogenes,* and *Salmonella paratyphi and found potential antibacterial activities (Swaminathan, 2018). The antimicrobial activity of I. tinctoria* against the above-mentioned microorganisms may due to the presence of phyto chemicals such as saponins, steroidal terpenes, anthroquinone, flavonoids, tannins and phenols as mentioned elsewhere in this report.

In addition, our study evaluated the antibiofilm activity of *I. tinctoria* methanolic crude extract against test organisms and showed potent antibiofilm efficiencies. The CAUTI is mainly related to biofilm which is observed on the surface of the catheter. As the biofilm formation is initiated when the microbes adhere to the solid surface and form colonization, our study focused on the effect of *I. tinctoria* on many of the results stages of biofilm formation and showed the effective antibiofilm activity of the plant extract by inhibiting the biofilm formation up to their MIC levels and it was again confirmed with eradication of mature biofilms of *E. faecalis, S. aureus, C. albicans, and E. coli* after the *I. tinctoria* treatment. Moreover, catheters provide the entry for many microbes to colonize on their surface resulting in severe complications. Hence, antimicrobial coating of catheter tubes is an excellent method to avoid the formation of biofilms. Therefore, the catheter tube was coated with methanolic extract of *I. tinctoria* and studied the potentials against *E. coli, S. aureus, E. faecalis,* and *C. albicans, in invitro* bladder model which mimicked the suitable environment and showed effective activity by forming clear zone around the catheter pieces. In support of this, a study (Jia *et al*.,2021) has reported that the catheter coating with polymer has shown potent efficiency against *S. aureus, E. coli,* and *Pseudomonas aeruginosa*. Another study conducted by Aleksandra *et al*. (2021) in a similar pattern has reported that the zinc oxide-coated catheter tube displayed efficiencies against *E. coli* and *S. aureus* for more than a week. There are other reports of studies with several coating agents such as silver nanoparticle and fosfomycin which showed excellent efficiencies against *E. coli, S. aureus,* and *E. faecalis* (Fisher *et al*., 2015; Rahuman *et al*., 2021; Abbott *et al*., 2020).

The present study, in addition, investigated the *I. tinctoria* methanolic crude extract for another pharmaceutical potential i.e., antioxidant property using DPPH, and showed excellent scavenging activity. In support of this, a report (*Swaminathan, 2018)* underlines the antioxidant properties of *I. tinctoria*. Additionally, the *I. tinctoria* methanolic extract was evaluated for its cytotoxicity against $L_{929}$ cells and showed no cytotoxic effect on normal cells indicating it can be used for human use.

## 5. Conclusion

The methanolic crude extract of *I. tinctoria* was evaluated for its antimicrobial and antibiofilm activities against *S. aureus, C. albicans, E. coli* and *E. faecalis* mainly involved in CAUTI. The extract showed excellent antimicrobial activities and effectively inhibited biofilm formation on non-living surfaces. Also, various concentrations of *I. tinctoria* effectively reduced the mature biofilms of test bacterial pathogens. The coating of the catheter tube with extract showed potent antibacterial against all the tested pathogens indicating its antiadhesive properties. In addition, the extract was looked at antioxidant properties and showed effective antioxidant properties and also, it was not cytotoxic to normal cells. The present study has limitations as it is just an *in vitro* investigation with a single solvent. Also, the study did not purify the extract to learn more about the compounds responsible for the antimicrobial activities. Overall, *I. tinctoria* is recommended for further purification for the phytocompounds responsible for the mentioned bioactivities for its preclinical trial for finding an alternative antimicrobial agent with a novel action mode against CAUTI infections.

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## Conflict of interest

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

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