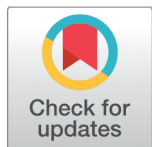


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Biofilm inhibition of UTI pathogens using *Terminalia arjuna* and *Ipomea carnea* plant extract

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Abstract

Objectives: To evaluate the antibiofilm potential of *Terminalia arjuna* and *Ipomea carnea* plant extract against potent biofilm forming UTI pathogens.

Methods/Statistical analysis: In this study, previously isolated and characterized three UTI pathogens viz. *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus equorum* were used to evaluate antibiofilm potential of selected plants. Soxhlet apparatus was used with a solvent methanol for the extraction process. For the evaluation of antibiofilm activity of different concentrations (2, 4, 6, 8 and 10 mg/ml) of plant extract in 3% DMSO Crystal violet assay was used. **Findings:** The methanolic bark extract of *T. arjuna* showed maximum activity up to 89.84% at 10mg/ml. against *S. equorum* while leaves extract of *I. carnea* gives maximum antibiofilm activity up to 67.53% at 2mg/ml against *E. coli*. **Applications:** The result shows that the investigated plants might be helpful in the development of potent herbal drugs to treat UTI.

Keywords: *T. arjuna*; *I. carnea*; *E. coli*; *P. aeruginosa*; *S. equorum*; Soxhlet extraction; Crystal violet assay

1 Introduction

Urinary tract infections (UTIs) are the most common human infectious disease affecting the bladder, kidneys and urinary tracts⁽¹⁾. *Escherichia coli* is the most frequent pathogen causing UTI in humans and one of the most common causes of Gram-negative bacteremia in hospitalized patients⁽²⁾. Other bacteria involved *Proteus mirabilis*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Enterococcus spp.*, *Enterobacter spp.*, group B *Streptococcus* and *S. saprophyticus*⁽³⁾. Biofilms that develop on indwelling devices like urinary catheters, pace-makers, voice prostheses, contact lenses, etc. are of major concern in medical practice⁽⁴⁾.

The cells embedded in a self-produced extracellular polymeric matrix produces microbial biofilms. They are the result of complex intra and inter cellular signaling and communication processes, regulated by a complex quorum sensing (QS) regulation system, which are ubiquitous in the microbial world⁽⁵⁾. Biofilm formation represents the prevailing microbial lifestyle in natural environments and occurs on all types of surfaces. Since biofilms interfere with the action of several antibiotics and bacterial drugs, biofilm-forming bacteria are more difficult to eradicate⁽⁶⁾.

Plant derived compounds have gained widespread interest in the search to identify the alternatives for microbial control⁽⁷⁾. Medicinal plants have been used for medicinal purposes worldwide, because of their significant health benefits. Various plant extract has great potential against infectious agents and can be used for therapeutic purposes⁽⁸⁾. The use of plant products, either as pure compounds or as standardized plant extracts, could be of great significance in the treatment of microbial diseases⁽⁹⁾.

Terminalia arjuna (Roxb.) Wight and Arn. (Combretaceae) is commonly known as Arjuna, found in Sub-Himalayan tract, Chota Nagpur, Orissa, West Bengal, Punjab, Deccan and Konkan⁽¹⁰⁾. The tree is about 60-80 feet height. Arjuna is large, evergreen with a spreading crown and dropping branches. In favorable localities and especially along the banks of streams, the tree attains very large sizes. Leaves sub-opposite, oblong or elliptic, coriaceous, cordate, shortly acute or obtuse at the apex. Flowers in panicle spikes. Fruits ovoid or ovoid-oblong, 2.5-5.0 cm long, nearly glabrous, with 5-7 hard, winged angles⁽¹¹⁾. The bark of *T. arjuna* is reported to act as anti-dysenteric, antipyretic, astringent, cardiogenic, lithotriptic, anticoagulant, hypolipidemic, antimicrobial agent⁽¹²⁾.

Bush Morning Glory botanically named as "*Ipomoea carnea* Jacq." from the family of Convolvulaceae. In India, it has become a naturalized species invading the wetlands, canals, drain banks, waste lands, field edges and road sides. The plant can propagate both vegetative by stems which show rooting within a few days and sexually by seed and has rapid growth rate⁽¹³⁾. This evergreen, flowering shrub grows to a height of 5m. The stem is thick and develops into a solid trunk over several years with many branches from base. The stem is erect, woody, hairy and more or less cylindrical in shape and greenish in color. It has alternate leaves. Normally it attains 1.25 - 2.75 m long and 0.5 - 0.8 cm diameter. The leaves are light green, heart shaped or somewhat lanceolate and 10-25cm long. The upper surface of leaf is dull green and the lower surface is paler. The leaves which receive lesser sunlight may grow larger than the leaves which receive full sunlight⁽¹⁴⁾. It possess antioxidant, antimicrobial, antibacterial, antifungal, anti-cancer, anti-convulsant, immune-modulator, anti-diabetic, hepato-protective, anticancer, anti-inflammatory, anxiolytic, sedative, wound healing and embryo toxic activities. Leaves are used as purgative. Leaves paste is applied on 'Haja'⁽¹⁵⁾.

The aim of this study was to investigate the potential antibiofilm activity of *T. arjuna* and *I. carnea* against pathogens associated with UTI.

2 Materials and Methods

2.1 Selection of plants

In the present study two medicinally important plants namely *Terminalia arjuna* and *Ipomea carnea* were screened and selected for the inhibition of biofilm formed by UTI pathogens.

2.2 Collection of plant materials

Fresh and healthy bark of *T. arjuna* and leaves of *I. carnea* were collected from different sites of Baramati (18.1792° N, 74.6078° E) to evaluate their anti-biofilm activity. The collected samples were transfer to laboratory and stored until use⁽¹⁶⁾.

2.3 Processing and extraction

Collected material was washed thoroughly in running tap water and plant parts were dried in shade at room temperature before grinding⁽¹⁷⁾.

2.3.1 Soxhlet extraction

The Soxhlet extractor setup consists of a round bottom flask, siphon tube, distillation path, expansion adapter, condenser, cooling water inlet, cooling water outlet, heat source and thimble. In this study, methanol was selected as a solvent. Total 150 ml of 99.5 % methanol was added in the round bottom flask followed by assembling the apparatus. The extraction process was carried out at 75°C temperature. The 15g of powder sample of the plant was placed in the thimble. The extraction was carried out for 24 hrs to complete 15 cycles. The extract collected was subjected to evaporation and dried extract were stored at low temperature until use⁽¹⁸⁾.

2.4 Evaluation of antibiofilm potential of plant extract

Five different concentrations (2, 4, 6, 8 and 10mg/ml) of plant extracts were prepared by dissolving in 3% DMSO. Freshly prepared 24 hrs old culture of the selected isolates in LB (Luria Bertani) broth was used for assay purpose. The cultures were serially diluted to 1:100 in fresh medium (Turbidity adjusted to 0.5 McFarland standards). The different concentrations of plant extracts were added in clean and sterile test tubes. One ml of 24 hrs old bacterial culture was added in each test tube. A positive control tube was treated with one ml of bacterial culture without plant extract while a negative control (Blank) tube was treated with one ml sterile broth without bacterial culture and plant extract. All the tubes incubated for 24 hrs at 37°C. After incubation, the unbound cells were removed by washing the tubes thrice with distilled water. The bound cells were then stained with 0.1% crystal violet solution for 20 min. Excess stain was rinsed off by thorough washing with distilled water and tubes were kept for air drying⁽¹⁹⁾. For the quantification of antibiofilm activity of plant extract, the adherent bacteria associated with crystal violet were solubilized with 30% acetic acid and the absorbance were recorded at 600nm using UV-Vis spectrophotometer. From the absorbance, the percentage of biofilm inhibition was calculated by the formula:

$$\% \text{ of inhibition} = \frac{OD \text{ of control} - OD \text{ of test}}{OD \text{ of control}} \times 100$$

All the experiments were carried out in triplicates. Standard error was determined.

In this study, previously isolated and characterized UTI pathogens such as *E. coli*, *P. aeruginosa* and *S. equorum* were successfully used for evaluation of antibiofilm potential of selected plants.

3 Results and Discussion

3.1 Selection of plants

Medicinal plants and their extracts are used in traditional treatments of various diseases⁽²⁰⁾. Plants are a potential source of antimicrobial compounds and several researchers throughout the world are investigating the antimicrobial activity of medicinal plants, which are utilized in the traditional or alternative healthcare systems⁽²¹⁾. Two important plant varieties namely *T. arjuna* and *I. carnea* were selected in the present study because they are very easily found in large no. in the region of Baramati, Pune (Maharashtra).

Chaudhari and Mahajan (2015) used stem bark and flavonoids of *T. arjuna* to check their antimicrobial and antibiofilm potential⁽²²⁾.

Mandal et al. (2013) also used bark of *T. arjuna* to check antifungal activity and they found that antimicrobial activity of *T. arjuna* bark extract showed greater result against Gram negative bacteria than Gram positive bacteria. Debnath et al. (2013) also reported the use of bark of *T. arjuna* to check antimicrobial activity against twenty two bacterial spp. including 8 uropathogens⁽¹²⁾.

Bhalerao et al. (2016) discussed about the antioxidant, anti-diabetic, antifungal, anti-inflammatory, anti-cancer, immunomodulatory, hepatoprotective, glycosidase inhibitory, anxiolytic, antimicrobial, cardiovascular, mosquitocidal and wound healing activity of *I. carnea*. According to them, *I. carnea* is one of the most promising shrubs which possess a lot of therapeutic values⁽²³⁾. Patel et al. (2014) checked anti-inflammatory activity of *I. carnea* plant leaves extract⁽²⁴⁾.

3.2 Collection of plant material

Bark and leaves sample from *T. arjuna* and *I. carnea* were selected respectively. The previous studies shows that the bark of *T. arjuna* have number of bioactive compounds with potential to act as antibiofilm agents and *I. carnea* are also reported to have various bioactivities^(22,23).

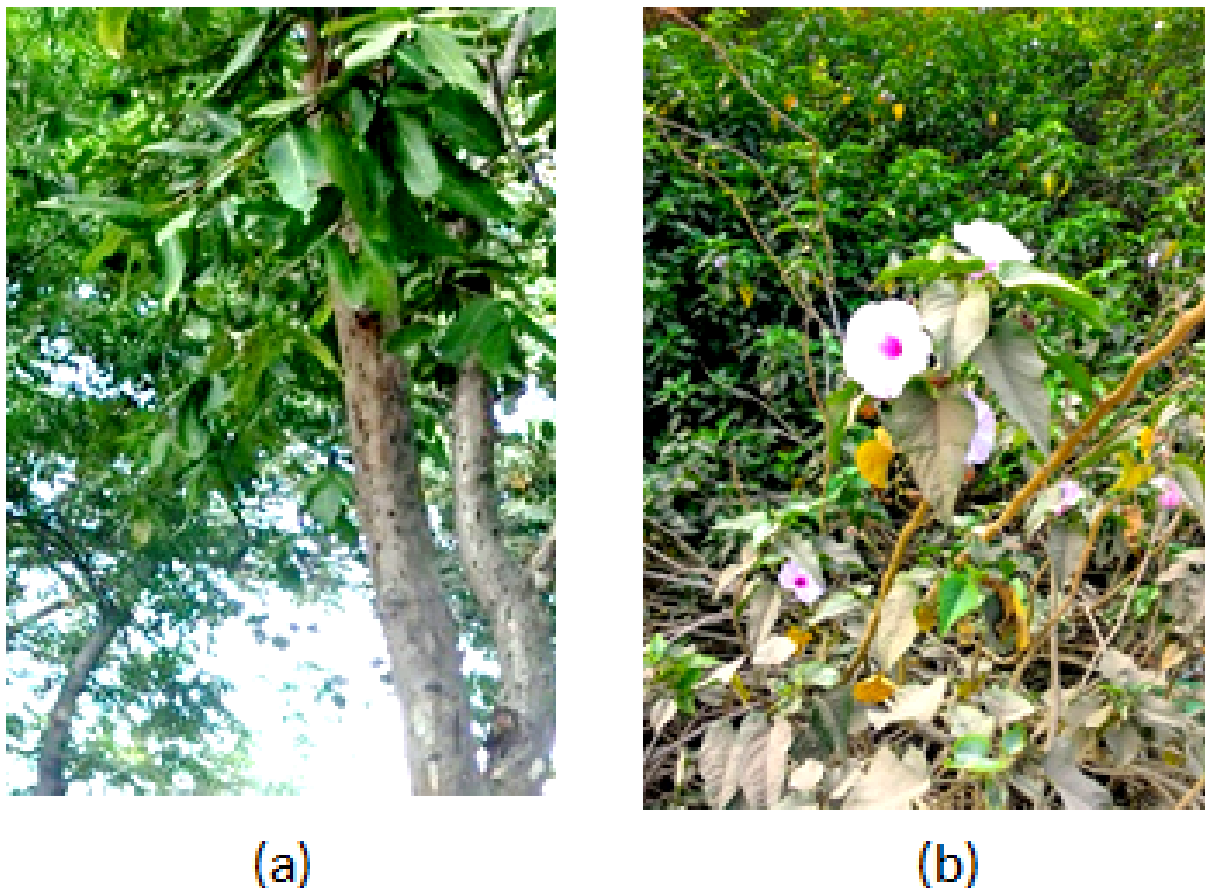


Fig 1. (a) *Terminalia arjuna* (Roxb.) Weight & Arn. (b) *Ipomea carnea* Jacq.

3.3 Processing and extraction

The collected samples were cleaned thoroughly by using distilled water to remove the impurities and biota. Bandiola (2018) also used similar procedure for cleaning of the plant material. According to them, the cleaning using the hands leads to better results and less damage on the plant. Wiping the samples with clean and dry cloth enhances the drying process⁽²⁵⁾.

After cleaning the samples were dried under shade for 3 weeks as shade drying has its own advantages. Mohsenipour et al. (2015)⁽²⁶⁾ used similar process of drying for *Allium sativum* bulb. They found that drying of the plant part in shade trigger the bioactive compounds of the sample.

We used mixer grinder in our study for size reduction, as the grinding helps to lowering particle size which increases surface contact between samples and extraction solvents⁽²⁶⁾. Vyas et al. (2018) also used electrical grinder. According to them, mixer grinding is more effective process but energy consuming when compared with mortar and pestle⁽²⁷⁾. Azwanida (2015) also discussed in their review about the conventional mortar and pestle and electric blenders and mills grinding⁽¹⁸⁾. Figure 2 (a-c) and Figure 3 (a-c) shows different stages of processing of collected

leaves samples from both the plants.



Fig 2. (a) Bark of *T. arjuna* before drying (b) Bark of *T. arjuna* after drying (c) Sample of *T. arjuna* after grinding



Fig 3. (a) Leaves of *I. carnea* before drying (b) Leaves of *I. carnea* after drying (c) Sample of *I. carnea* after grinding

3.3.1 Soxhlet extraction

The temperature resistant plant samples are generally subjected to hot extractions, so in the present study, we used Soxhlet extraction method for the extraction purpose. After 15 cycles of the extraction, the solvent was evaporated successfully to obtain a pure dried extract. The methanol is widely used for extraction of bioactive compounds from plant samples, as it is suitable for extraction of more polar compounds.

Azwanida (2015) discussed about the Soxhlet extraction techniques along with other conventional techniques used for extraction purpose. According to them, this method requires a smaller quantity of solvent compared to maceration but the Soxhlet extraction comes with disadvantage such as exposure to hazardous and flammable liquid organic solvents, with potential toxic emissions during extraction⁽¹⁸⁾.

According to De Castro et al. (1998) most of these conventional methods have in common with Soxhlet. According to them, they are time-consuming and require large amount of solvent. On the other hand, they are relatively simple both in performance and fundamentals, so their development does not require specialized personnel. These methods are cheap, which has favored their widespread use particularly both in industries and routine laboratories⁽²⁸⁾.



Fig 4. Soxhlet extraction of (a) *T. arjuna* bark and (b) *I. carnea* leaves

3.4 Evaluation of antibiofilm potential of plant extract

Crystal violet assay was successfully used for determining biofilm inhibition potential of plant extract against selected three isolates. For this purpose, five different concentrations (2, 4, 6, 8 and 10 mg/ml) of extract was used. The quantitative estimation of biofilm inhibition was done after washing by the spectrophotometric (600nm) assay. % inhibition of biofilm was determined at each concentrations by using standard formula.

3.4.1 Evaluation of antibiofilm activity of *T. arjuna*

Table 1 shows % inhibition of biofilm produce by these three organisms at five different concentrations.

By using % inhibition formula, the value obtained showed that *E. coli* have highest sensitivity to 8 mg/ml (74.25%) concentrations of the extract followed by 10 mg/ml (64.91%), 6 mg/ml (64.11%), 2 mg/ml (54.45%) and 4 mg/ml (49.16%). These results showed that there was no linear relationship, further increase or decrease in concentrations did not showed significant effect on antibiofilm potential of the isolates.

The different concentrations of the extract tested for antibiofilm activity evaluation found that *P. aeruginosa* showed highest sensitivity to *T. arjuna* extract at 10 mg/ml (46.33%) followed by 6 mg/ml (41.96%), 8 mg/ml

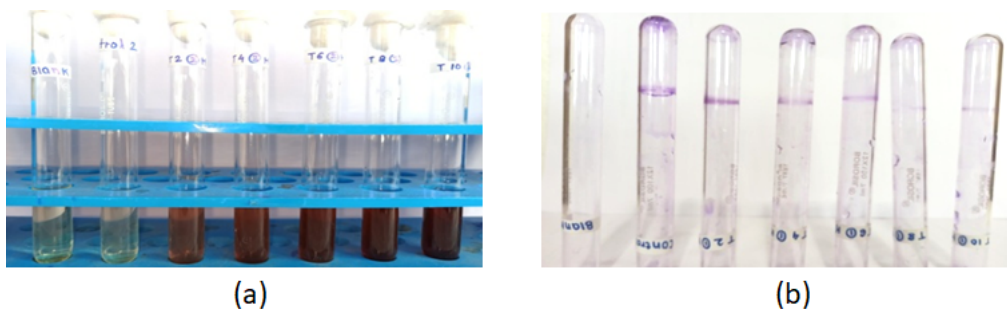


Fig 5. (a) Effect of different concentration of *T. arjuna* extract on biofilm formation of *E.coli* (b) Antibiofilm activity of *T. arjuna* against *E. coli* by crystal violet assay

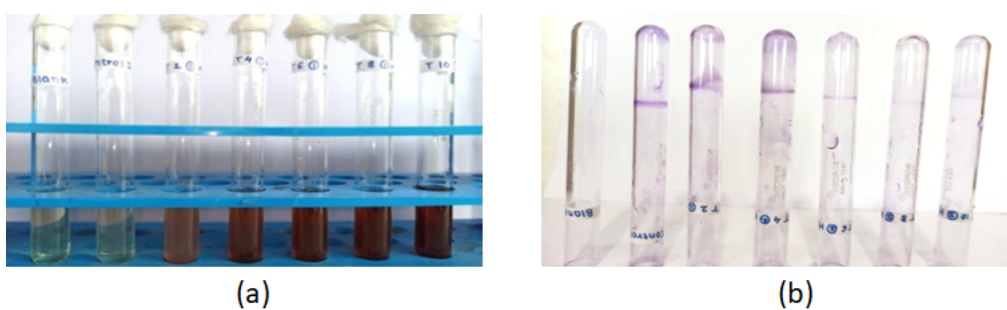


Fig 6. (a) Effect of different concentration of *T. arjuna* extract on biofilm formation of *P. aeruginosa* (b) Antibiofilm activity of *T. arjuna* against *P. aeruginosa* by crystal violet assay

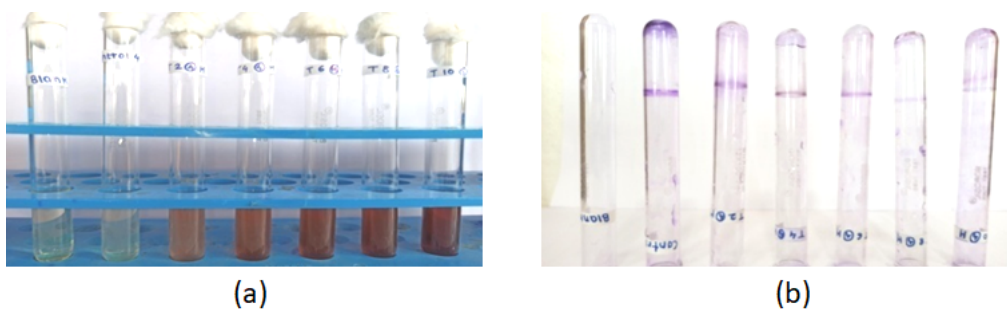


Fig 7. (a) Effect of different concentration *T. arjuna* extract on biofilm formation of *S. equorum* (b) Antibiofilm activity of *T. arjuna* against *S. equorum* by crystal violet assay

Table 1. Antibiofilm activity of the *T. arjuna* against three isolates by crystal violet assay

Organisms	% Inhibition				
	2 mg/ml	4 mg/ml	6 mg/ml	8 mg/ml	10 mg/ml
<i>E. coli</i>	54.45±4.08	49.16±4.56	64.11±5.23	74.25±6.40	64.91±5.27
<i>P. aeruginosa</i>	20.49±7.86	20.96±6.27	41.94±5.87	23.83±4.69	46.33±5.35
<i>S. equorum</i>	66.99±4.86	85.86±5.39	87.43±6.05	88.42±4.50	89.84±5.20

*Mean values ± Standard error of means

(23.83%) and 4 mg/ml (20.96%). The lowest antibiofilm activity was observed at 2 mg/ml (20.49%).

S. equorum also showed sensitivity to the different concentrations of the extract, maximum activity was observed at 10 mg/ml (89.84%) followed by 8 mg/ml (88.42%), 6 mg/ml (87.43%) and 4 mg/ml (85.86%) and 2 mg/ml (66.99%). As extract concentrations increase, % inhibition was also found to increase.

3.4.2 Evaluation of antibiofilm activity of *I. carnea*

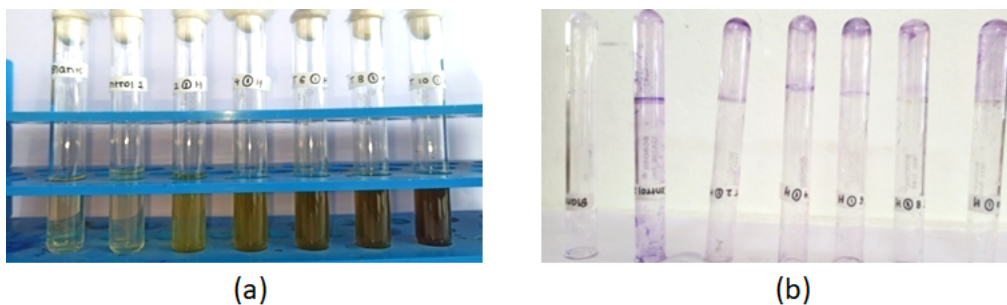


Fig 8. (a) Effect of different concentration of *I. carnea* extract on biofilm formation of *E. coli* (b) Antibiofilm activity of *I. carnea* against *E. coli* by crystal violet assay

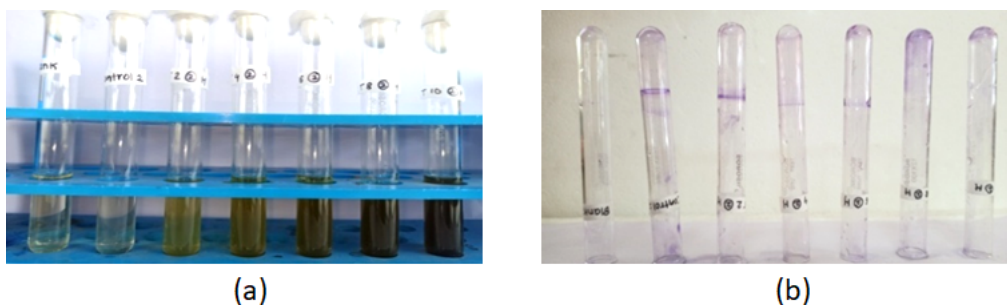


Fig 9. (a) Effect of different concentration of *I. carnea* extract on biofilm formation of *P. aeruginosa* (b) Antibiofilm activity of *I. carnea* against *P. aeruginosa* by crystal violet assay

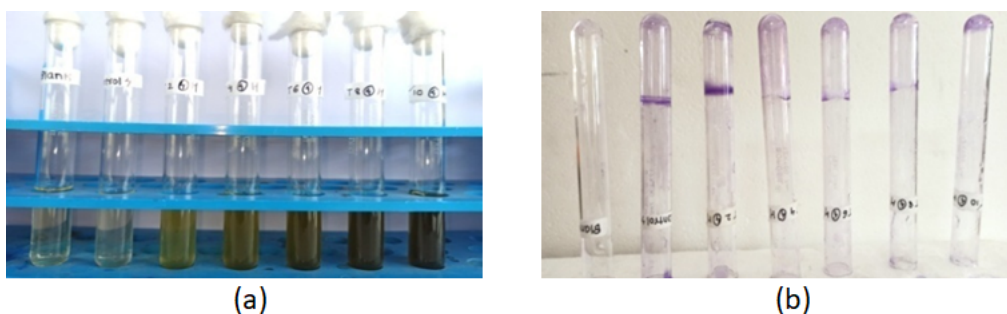


Fig 10. (a) Effect of different concentration of *I. carnea* extract on biofilm formation of *S. equorum* (b) Antibiofilm activity of *I. carnea* against *S. equorum* by crystal violet assay

Table 2 shows % inhibition of biofilm produce by these three organisms at five different concentrations.

Table 2. Antibiofilm activity of the *I. carnea* against three isolates by crystal violet assay

Organisms	% Inhibition				
	2 mg/ml	4 mg/ml	6 mg/ml	8 mg/ml	10 mg/ml
<i>E. coli</i>	67.53±6.44	62.24±4.14	61.82±4.84	65.54±5.32	51.34±7.38
<i>P. aeruginosa</i>	17.41±7.87	17.49±6.46	30.64±6.49	8.78±5.43	23.66±4.78
<i>S. equorum</i>	11.04±4.9	21.6±6.58	50.10±7.22	42.5±4.21	49.50±4.60

*Mean values ± Standard error of means

E. coli showed highest sensitivity to 2 mg/ml (67.53%) concentrations of the extract followed by 8 mg/ml (65.54%), 4 mg/ml (62.24%), 6 mg/ml (61.82%) and 10 mg/ml (51.34%). There was no linear relationship present in extract concentrations and antibiofilm activity.

P. aeruginosa showed highest sensitivity to *I. carnea* extract at 6 mg/ml (30.64%) followed by 10 mg/ml (23.66%), 4 mg/ml (17.49%) and 2 mg/ml (17.41%). The lowest antibiofilm activity was observed at 8 mg/ml (8.78%). *S. equorum* also showed sensitivity to the different concentrations of the extract but it was found that maximum activity was observed at 6 mg/ml (50.10%) followed by 10 mg/ml (49.50%), 8 mg/ml (42.5%) and 4 mg/ml (21.6%) and 2 mg/ml (11.04%).

All the experiments are carried out in triplicates. Standard error was successfully calculated and showed by error bars in the following Figure 11 (a & b).

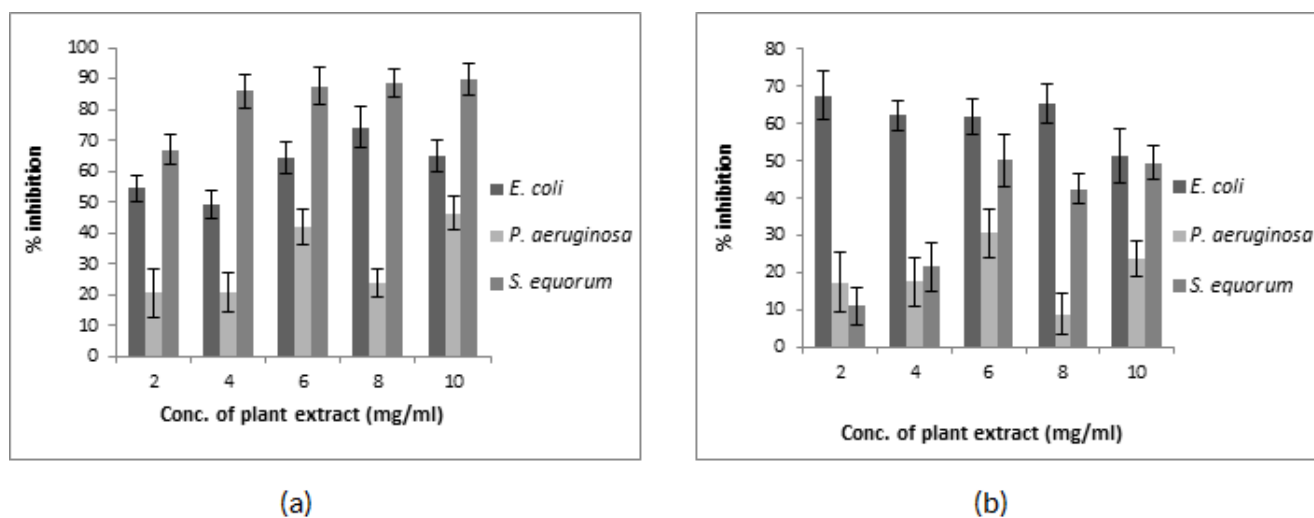


Fig 11. Antibiofilm activity of (a) *T. arjuna* and (b) *I. carnea* extract against isolates.

Chaudhari and Mahajan (2015) studied the antimicrobial and antibacterial potential of *T. arjuna* against *E. coli* and *S. aureus*. The results showed that plant extract are able to inhibit biofilm formation and they are important resource of biofilm inhibitors compounds⁽²²⁾.

According to Packiavathy et al. (2014) *Curcuma longa* plant extract have the antibiofilm, antifungal, antibacterial and antiviral activity. According to them, the plant extract of *Curcuma longa* shows 81% and 68% maximum antibiofilm activity against *E. coli* and *P. aeruginosa* respectively⁽²⁹⁾.

Etim et al. (2016) reported antibacterial potential of *B. pinnatum* against *Staphylococcus* spp⁽³⁰⁾.

Famuyide et al. (2019) studied antibiofilm activity of nine *Eugenia* and *Syzygium* (Myrtaceae) spp. Among these *S. legatii* showed highest biofilm inhibition 100% against *E. coli* and *E. umtamvunensis* showed highest biofilm inhibition upto 83% against *P. aeruginosa*⁽³¹⁾.

Romero et al. (2016) reported antibiofilm potential of *L. chilense*, *T. minuta*, *T. absinthioides* and *L. divaricata* against *Staphylococcus spp.* *L. chilense* showed highest antibiofilm activity upto 68%. *T. minuta*, *T. absinthioides* and *L. divaricata* showed antibiofilm activity in between 55% to 62%⁽³²⁾.

4 Conclusion

The UTI pathogens show resistant to different antibiotics, which makes these infections more serious. The biofilm forming ability of these pathogens play major role in pathogenesis. Hence, antibiofilm agents can be effectively used against them.

The bioactive compounds from plant extracts of *T. arjuna* and *I. carnea* showed notable effect on biofilms and can be potentially used in the treatment of UTI. The inhibition of biofilm formation of *E.coli*, *P. aeruginosa* and *S. equorum* was shown by all the tested concentrations of plant extracts in dose dependent manner. The study conducted concludes, the bioactive compounds from *T. arjuna* and *I. carnea* can be used as potential antibiofilm agents against UTI infection.

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