World Journal of Pharmaceutical and Life Sciences
WJPLS
www.wjpls.org
SJIF Impact Factor: 7.409

ANTIBIOFILM ACTIVITY OF CITRIC ACID AGAINST DUAL BACTERIAL BIOFILMS

***Dr. Anuya Aparna Rege**

Head, Bacteriology Department, Haffkine Institute for Training, Research and Testing, Parel, Mumbai- 400012, India.

***Corresponding Author: Dr. Anuya Aparna Rege**
Head, Bacteriology Department, Haffkine Institute for Training, Research and Testing, Parel, Mumbai- 400012, India.

Article Received on 11//06/2025 **Article Revised on 01/07/2025** **Article Accepted on 22/07/2025**

ABSTRACT
Both *E. coli* and *Salmonella* species could adhere to various food-contact surfaces and produce biofilms leading to recurrent contamination and foodborne outbreaks. Nearly all biofilms found in nature are made up of mixed species. Therefore, there is urgent need for effective antibiofilm agent targeting multi-bacterial biofilms. Hence, in the present *in vitro* study, Citric Acid was evaluated for its Antibiofilm potential against Dual Bacterial Biofilms of *E. coli*, *S. enterica* and *S. typhi* with different bacteria using crystal violet staining method. Citric Acid exerted the highest antibiofilm effect against Dual bacterial biofilms of *E. coli* + *S. enterica* followed by *E. coli* + *E. faecalis* > *E. coli* + *MRSA* > *E. coli* + *S. typhi* > *E. coli* + *P. aeruginosa* > *S. enterica* + *MRSA* > *S. typhi* + *MRSA* > *S. typhi* + *E. faecalis* > *S. enterica* + *E. faecalis* > *S. enterica* + *S. typhi* > *S. enterica* + *P. aeruginosa* > *S. typhi* + *P. aeruginosa*. Combination prepared from Citric Acid (CA) and Ciprofloxacin (CP) was also evaluated against total 12 Dual bacterial biofilms that showed somewhat similar antibiofilm activity to that of Citric Acid alone. In our previous studies, Citric Acid has displayed antibiofilm potential against Mono and Mixed Bacterial biofilms as well as against Dual and Polymicrobial biofilms of *C. albicans* with different Bacteria [Fungus + Bacteria], whereas, in present *in vitro* study, Citric Acid has revealed antibiofilm potential against Dual bacterial biofilms [Bacteria + Bacteria]. Therefore, Citric Acid could be used as an effective antibiofilm agent to treat microbial biofilms related menaces be it foodborne diseases or nosocomial infections.

KEYWORDS: Citric Acid, Antibiofilm, Natural Products, Antibacterial, Biofilm Assay.

INTRODUCTION

Biofilms are surface-adherent aggregates of bacterial communities embedded within an extracellular, self-produced polymeric matrix.[1] Bacterial biofilm formation is considered to be an emergent and prevailing microbial lifestyle in natural and manmade environments and occurs on all surface types. Food and food processing environments are the best sites for microbial attachment and biofilm formation.[2] Biofilms account for approximately 60% of foodborne outbreaks.[3] Biofilm formation confers numerous advantages to microbial cells in food industry environment, such as physical resistance against desiccation, mechanical resistance against liquid streams in pipelines and chemical protection against chemicals, antimicrobials and disinfectants used in the industry.[4] Both *E. coli* and *Salmonella* species could adhere to various food-contact surfaces and produce biofilms which could function as barrier against disinfectants and promote bacterial tolerance toward sanitizers. Nearly all biofilms found in nature are made up of mixed species and the intricate interactions within the biofilms have a substantial impact on the biofilm structure and biological activity.[5] Hence, treatment of poly-microbial biofilms require antimicrobials that are effective against all microorganisms in the biofilms which creates an additional challenge.[6] Natural compounds are regarded as safe and biodegradable and may penetrate the biofilm structure killing the microbes in it. Application of Citric acid is one such alternatives. Citric Acid is a weak organic (tricarboxylic) acid found in fruits such as limes, lemons, blackberries, grapefruits as well as oranges, raspberries and strawberries. Citric Acid was first time isolated from lemon juice in 1784 by Carl Scheele, whereas, industrial scale production of Citric Acid involving microorganisms was initiated in 1917 by Currie, who developed a method for obtaining it from filamentous fungi. Citric Acid has found wide application in various industrial sectors such as pharmaceutical, chemical and food industries, due to its biocompatibility, versatility and environmentally friendly chemistry. Its three carboxylic groups and one hydroxyl group provide the functionality and versatility required for its many applications.[7,8] Our earlier study has revealed antibiofilm potential of Citric Acid against Mono-species bacterial biofilms.[9] Therefore, in the

present *in vitro* study, Citric Acid was further evaluated for its Antibiofilm potential against Dual-species biofilms of *E. coli, S. enterica* and *S. typhi* with different Bacteria.

MATERIALS AND METHODS

1. Preparation of Test Solution

The test solution was prepared by dissolving Citric Acid, Anhydrous, Extrapure (SRL) in sterile distilled water (Stock solution= 100 mg/ml). The prepared test solution was stored at 4^0C until further use.

2. Test Organisms used for Antibiofilm Assays

Antibiofilm activity of Citric Acid was evaluated against biofilms of dual bacterial species. For this, *Escherichia coli* (Hb-101), *Pseudomonas aeruginosa* (Fisher's Immuno Type-IV), *Salmonella enterica* (NCIM-5256), *Salmonella typhi* (NCTC-786), *Enterococcus faecalis* (ATCC-29212) and Methicillin Resistant *Staphylococcus aureus* (*MRSA* ATCC-25923) were included in the said study. Bacterial cultures were grown on Nutrient agar and suspended in Mueller Hinton Broth (MHB) for the assays.

3. Anti-Biofilm Assay against Dual Species Biofilms (Bacteria + Bacteria)

Effect of Citric Acid was evaluated on Dual species Biofilms of *E. coli, S. enterica* and *S. typhi* with different bacteria using Crystal Violet staining method as stated earlier.[9] For this study, different concentrations of Citric Acid such as 0.1mg/ml, 1mg/ml and 10mg/ml were used. Ciprofloxacin (2 mg/ml) was included as standard antibiotic in the said study. The microplates were incubated at 37^0C for 48h. After incubation, supernatant was removed and each well was washed thoroughly with sterile distilled water thrice to remove free-floating cells; thereafter plates were air-dried for 30 min and the biofilm formed was stained with 0.1% aqueous solution of crystal violet for 15 min at room temperature. Following incubation, the excess of stain was removed washing the plate three times with sterile distilled water. Finally, the dye bound to the cells were solubilized by adding 250 μl of 95% ethanol to each well and after 15 min of incubation, absorbance was measured using Multimode Reader (Synergy HT, BioTek) at a wavelength of 570 nm. Effect on dual bacterial Biofilms was determined using the formula Percentage Inhibition = (Control − Test)/Control X 100, where Control is the OD_{570nm} of the stained Control wells containing distilled water and Test is the OD_{570nm} of the stained Test wells containing Citric Acid or Ciprofloxacin (standard) respectively.

Following 12 dual species biofilms prepared in 1:1 ratio were included in the said study,

- ☐ *E. coli + P. aeruginosa*
- ☐ *E. coli + S. enterica*
- ☐ *E. coli + S. typhi*
- ☐ *E. coli + E. faecalis*
- ☐ *E. coli + MRSA*
- ☐ *S. enterica + P. aeruginosa*
- ☐ *S. enterica + S. typhi*
- ☐ *S. enterica + E. faecalis*
- ☐ *S. enterica + MRSA*
- ☐ *S. typhi + P. aeruginosa*
- ☐ *S. typhi + E. faecalis*
- ☐ *S. typhi + MRSA*

Note: The peripheral wells of the microplates were filled with sterile Distilled Water to avoid edge effect. Further, the plates were placed in a tray and then kept in an incubator for the incubation to prevent loss of contents due to evaporation.

4. Combinatorial Effect on Biofilms of Dual-Bacterial species (Bacteria + Bacteria)

Combinatorial effect of Citric Acid (10mg/ml) with Standard Antibiotic Ciprofloxacin (2 mg/ml) was evaluated against above mentioned 12 Biofilms of Dual-bacterial species. The microplates were incubated at 37^0C for 48h. After incubation, supernatant was removed and the effect of combination solution on biofilms of dual-bacterial species was determined by Crystal Violet staining method as stated earlier. Combination of Citric Acid + Ciprofloxacin (CA+CP) prepared in 1:1 ratio was included in the said study.

Note: The peripheral wells of the microplates were filled with sterile Distilled Water to avoid edge effect. Further, the plates were placed in a tray and then kept in an incubator for the incubation to prevent loss of contents due to evaporation.

RESULTS

Table 1: Effect of Citric Acid on Dual Bacterial Biofilms of *E. coli*.

Test Solution	Concentration (mg/ml)	Inhibition (%)				
		E. coli* + *P. aeruginosa	***E. coli* + *S. enterica***	***E. coli* + *S. typhi***	***E. coli* + *E. faecalis***	***E. coli* + *MRSA***
Citric Acid	0.1	Nil	Nil	Nil	19.75	Nil
	1	5.17	25.51	Nil	37.96	Nil
	10	82.95	91.15	85.86	90.99	88.85
Ciprofloxacin	2	79.60	90.41	85.20	91.17	89.47

***Note**: Mean of triplicate determinations*

Table 2: Effect of Citric Acid on Dual Bacterial Biofilms of *S. enterica*.

Test Solution	Concentration (mg/ml)	Inhibition (%)			
		S. enterica* + *P. aeruginosa	***S. enterica* + *S. typhi***	***S. enterica* + *E. faecalis***	***S. enterica* + *MRSA***
Citric Acid	0.1	Nil	Nil	Nil	2.04
	1	26.84	Nil	Nil	Nil
	10	58.85	74.27	74.65	78.61
Ciprofloxacin	2	66.40	77.02	78.05	80.65

***Note**: Mean of triplicate determinations*

Table 3: Effect of Citric Acid on Dual Bacterial Biofilms of *S. typhi.*

Test Solution	Concentration (mg/ml)	Inhibition (%)		
		S. typhi* + *P. aeruginosa	***S. typhi* + *E. faecalis***	***S. typhi* + *MRSA***
Citric Acid	0.1	Nil	Nil	Nil
	1	Nil	3.58	Nil
	10	50.58	76.38	77.91
Ciprofloxacin	2	51.73	73.94	78.34

***Note**: Mean of triplicate determinations*

Table 4: Effect of Citric Acid + Ciprofloxacin combination on Dual Bacterial Biofilms.

No.	Dual Bacterial Biofilms	Inhibition (%)
1	*E. coli* + *P. aeruginosa*	82.28
2	*E. coli* + *S. enterica*	90.75
3	*E. coli* + *S. typhi*	84.45
4	*E. coli* + *E. faecalis*	89.69
5	*E. coli* + *MRSA*	87.61
6	*S. enterica* + *P. aeruginosa*	65.01
7	*S. enterica* + *S. typhi*	75.73
8	*S. enterica* + *E. faecalis*	76.51
9	*S. enterica* + *MRSA*	78.47
10	*S. typhi* + *P. aeruginosa*	48.27
11	*S. typhi* + *E. faecalis*	74.43
12	*S. typhi* + *MRSA*	75.87

***Note**: Mean of triplicate determinations*

DISCUSSION

Microbial biofilms which are communities of bacteria embedded in self-produced matrix, pose significant challenges in food processing environments. Common biofilm-forming bacteria in food processing facilities include *Salmonella* and *E. coli*. These pathogens can persist in food processing plants leading to recurrent contamination and outbreaks.[10] Most natural biofilms are actually formed by multiple bacterial species and these multispecies biofilms are dynamic communities with extensive interactions between species.[11] Therefore, there is urgent need for effective antibiofilm agent targeting multispecies biofilms. Citric Acid could be a possible source of effective and safe candidate for eradication of poly-microbial biofilms. Hence, in the present *in vitro* study, Citric Acid was evaluated for its Antibiofilm potential against biofilms of dual bacterial species (Bacteria + Bacteria) using crystal violet staining method.

Crystal violet staining for biofilm quantification remains the most frequently used quantification technique in microtitre plate assays. These assays stain both live and dead cells as well as some components present in biofilm matrix, thereby being well suited to quantify total biofilm biomass. After staining, the adsorbed crystal violet is eluted using ethanol or acetic acid. The amount of dye solubilized by the solvent (measured by optical absorbance) is directly proportional to the biofilm size. The method can be used with broad range of different bacterial species as well as yeasts or fungi. It also offers high throughput capability of the method, allowing testing of many different conditions simultaneously.[12,13]

Likewise, the microtiter plate assay is an important tool for the study of the early stages in biofilm formation. It allows rapid testing of a large number of compounds in a single run, using very small amount of tested compounds. The scientific community has extensively used microplates to grow bacterial biofilms. This simple microtiter plate assay allows the formation of a biofilm on the wall and/or bottom of a microtiter plate. The biofilm formation is measured using the dye crystal violet.[14,15] However, microtiter plate-based assays share issue of “Edge Effect”. The “Edge Effect” poses serious concerns when antimicrobial efficacy of compounds is to

be determined, as due to evaporation, concentration of "testing compound" increases which gives false crystal violet absorbance values.[16] To reduce excessive content loss and to maintain humidity, adding autoclaved water to peripheral wells and placing the sealed microplates in a tray considerably reduced the edge effect in the present study.

In order to the biofilms to form in the presence of test solutions, the planktonic cells would need to survive the test solution concentrations long enough to permit attachment. Therefore, this assay measures both cell attachment and biofilm proliferation in presence of test solutions. In the present study, Citric Acid was evaluated against total 12 dual bacterial biofilms of *E. coli*, *S. enterica* and *S. typhi* with different Bacteria. For which, three different concentrations of Citric Acid mainly, 0.1mg/ml, 1mg/ml and 10mg/ml were included in the Anti-biofilm assay in the present study. Among the dual bacterial biofilms of *E. coli*, Citric Acid showed the highest antibiofilm activity at the concentration of 10mg/ml against dual biofilm of *E. coli* + *S. enterica* followed by *E. coli* + *E. faecalis* > *E. coli* + *MRSA* > *E. coli* + *S. typhi* > *E. coli* + *P. aeruginosa*. Further, dose dependent antibiofilm activity of Citric Acid was observed against dual biofilm of *E. coli* + *E. faecalis*, i.e, inhibitory activity increased with increasing concentration [Table-1].

Ciprofloxacin was included as positive control in the said study, wherein, Ciprofloxacin displayed the highest antibiofilm effect against dual bacterial biofilm of *E. coli* + *E. faecalis* followed by *E. coli* + *S. enterica* > *E. coli* + *MRSA* > *E. coli* + *S. typhi* > *E. coli* + *P. aeruginosa* [Table-1].

Among the dual species biofilms of *S. enterica*, Citric Acid displayed the highest antibiofilm effect at the concentration of 10mg/ml in the following order, *S. enterica* + *MRSA* > *S. enterica* + *E. faecalis* > *S. enterica* + *S. typhi* > *S. enterica* + *P. aeruginosa* [Table-2]. Ciprofloxacin which was included as positive control in the said study exerted antibiofilm effect in similar order that of Citric Acid [Table-2].

Besides, amongst the dual species biofilms of *S. typhi*, Citric Acid displayed the highest antibiofilm activity at the concentration of 10mg/ml against *S. typhi* + *MRSA* followed by *S. typhi* + *E. faecalis* > *S. typhi* + *P. aeruginosa* [Table-3]. Ciprofloxacin which was included as positive control in the said study exhibited antibiofilm effect in similar order that of Citric Acid [Table-3].

In general, Citric Acid exerted the antibiofilm effect against total 12 dual bacterial biofilms in the following order, *E. coli* + *S. enterica* (91.15%) > *E. coli* + *E. faecalis* > *E. coli* + *MRSA* > *E. coli* + *S. typhi* > *E. coli* + *P. aeruginosa* > *S. enterica* + *MRSA* > *S. typhi* + *MRSA* > *S. typhi* + *E. faecalis* > *S. enterica* + *E. faecalis* > *S. enterica* + *S. typhi* > *S. enterica* + *P. aeruginosa* > *S. typhi* + *P. aeruginosa* (50.58%).

Combining antibiofilm agents with antibiotics is emerging as a promising strategy to eradicate biofilms.[17] Natural antimicrobial agents can act alone or in combination with standard antibiotics to enhance antimicrobial activity against a wide range of microbes.[18] Hence, combination prepared from Citric Acid (CA) and Ciprofloxacin (CP) the standard antibiotic was evaluated to determine its combinatorial effect against total 12 dual bacterial biofilms of *E. coli*, *S. enterica* and *S. typhi* with different Bacteria using crystal violet staining method. Overall, the combinatorial effect of CA+CP against total 12 dual bacterial biofilms was noted as follows, *E. coli* + *S. enterica* (90.75%) > *E. coli* + *E. faecalis* > *E. coli* + *MRSA* > *E. coli* + *S. typhi* > *E. coli* + *P. aeruginosa* > *S. enterica* + *MRSA* > *S. enterica* + *E. faecalis* > *S. typhi* + *MRSA* > *S. enterica* + *S. typhi* > *S. typhi* + *E. faecalis* > *S. enterica* + *P. aeruginosa* > *S. typhi* + *P. aeruginosa* (48.27%) [Table-4].

Generally, in case of dual species biofilms of *E. coli* + *P. aeruginosa* and *S. typhi* + *E. faecalis*, the combination CA+CP displayed decreased antibiofilm activity as compared to Citric Acid alone. However, it showed increased antibiofilm activity as compared to Ciprofloxacin alone. Moreover, the combination CA+CP exerted slightly decreased antibiofilm effect against dual species biofilms of *E. coli* + *S. enterica*, *E. coli* + *S. typhi*, *E. coli* + *E. faecalis*, *E. coli* + *MRSA*, *S. enterica* + *MRSA*, *S. typhi* + *P. aeruginosa* and *S. typhi* + *MRSA* as compared to individual testing solutions, viz., Citric Acid and Ciprofloxacin respectively. Besides, the combination CA+CP showed increased antibiofilm activity against dual species biofilms of *S. enterica* + *P. aeruginosa*, *S. enterica* + *S. typhi* and *S. enterica* + *E. faecalis* as compared to Citric Acid independently, whereas, the combination exhibited somewhat decreased antibiofilm effect as compared to Ciprofloxacin individually.

The interactions among multi-species biofilms are generally cooperative, competitive or neutral and these interactions among multiple species biofilms are effected by the environment and their own properties. The competitive and cooperative interactions in multi-species biofilms can promote resistance to antimicrobial agents. However, these interactions among different strains in multi-species biofilms may also have no positive impact on the disinfection resistance of microorganisms.[11,19]

Overall, in case of dual species biofilms of *E. coli* + *P. aeruginosa*, the total biomass of this dual biofilm was found to be decreased as compared to biomass of individual biofilms and the total biomass of this dual species biofilm was also less than average biomass of both the biofilms. In case of dual species biofilms of *E. coli* + *S. enterica*, the total biomass of this dual species biofilm was found to be substantially increased as compared to biomass of *E. coli* biofilm and *S. enterica*

biofilm individually. Further, the total biomass of this dual species biofilm was found to be more than the average biomass of both the biofilms. In case of dual species biofilms of *E. coli* + *S. typhi*, the total biomass of this dual species biofilm was found to be decreased as compared to biomass of individual biofilms and the total biomass of this dual species biofilm was also less than average biomass of both the biofilms. In case of dual species biofilms of *E. coli* + *E. faecalis*, the total biomass of this dual species biofilm was found to be substantially increased as compared to biomass of *E. coli* biofilm and *E. faecalis* biofilm individually. Furthermore, the total biomass of this dual species biofilm was found to be more than the average biomass of both the biofilms. Likewise, in case of dual species biofilms of *E. coli* + *MRSA*, the total biomass of this dual species biofilm was found to be substantially increased as compared to biomass of *E. coli* biofilm and *MRSA* biofilm independently. Furthermore, the total biomass of this dual species biofilm was found to be more than the average biomass of both the biofilms. Besides, in case of dual species biofilms of *S. enterica* + *P. aeruginosa*, the total biomass of this dual species biofilm was found to be decreased as compared to biomass of *S. enterica* biofilm and substantially decreased as compared to biomass of *P. aeruginosa* biofilm alone and the total biomass of this dual species biofilm was also less than average biomass of both the biofilms. Similarly, in case of dual species biofilms of *S. enterica* + *S. typhi*, the total biomass of this dual species biofilm was found to be decreased as compared to biomass of *S. enterica* biofilm and even substantially decreased as compared to biomass of *S. typhi* biofilm and the total biomass of this dual species biofilm was also less than average biomass of both the biofilms. In case of dual species biofilms of *S. enterica* + *E. faecalis*, the total biomass of this dual species biofilm was found to be increased slightly as compared to biomass of both the biofilms individually and the total biomass of this dual species biofilm was also more than average biomass of both the biofilms. Likewise, in case of dual species biofilms of *S. enterica* + *MRSA*, the total biomass of this dual species biofilm was found to be increased slightly as compared to biomass of both the biofilms individually and the total biomass of this dual species biofilm was also more than average biomass of both the biofilms. Furthermore, in case of dual species biofilms of *S. typhi* + *P. aeruginosa*, the total biomass of this dual species biofilm was found to be substantially decreased as compared to biomass of both the biofilms independently and the total biomass of this dual species biofilm was also less than average biomass of both the biofilms. In case of dual species biofilms of *S. typhi* + *E. faecalis*, the total biomass of this dual species biofilm was found to be considerably decreased as compared to biomass of *S. typhi* biofilm, whereas, the total biomass of this dual species biofilm was found to be increased as compared to biomass of *E. faecalis* biofilm and the total biomass of this dual species biofilm was less than average biomass of both the biofilms. Similarly, in case of dual species biofilms of *S. typhi* + *MRSA*, the total biomass of this dual species biofilm was found to be substantially decreased as compared to biomass of *S. typhi* biofilm, whereas, the total biomass of this dual species biofilm was found to be increased as compared to biomass of *MRSA* biofilm and the total biomass of this dual species biofilm was less than average biomass of both the biofilms. Li Q *et al.*[19] have stated that the competitive interaction between *Salmonella* and *P. aeruginosa* would result in decrease in the density of dual-species biofilms. The present *in vitro* study has noted similar observations.

In general, Citric Acid has revealed Antibiofilm potential against Dual biofilms of *E. coli*, *S. enterica* and *S. typhi* with different bacteria in present *in vitro* study. The mechanism of Citric Acid's strong antibacterial action could be related to acidifying the cytoplasm, disrupting metabolic processes or accumulating the dissociated acid anion to a toxic level.[7]

In our previous studies, Citric Acid has displayed antibiofilm potential against Mono and Mixed-Bacterial Species[9] as well as against Dual and Polymicrobial biofilms of *C. albicans* with different Bacteria (Fungus + Bacteria)[20], whereas, in present *in vitro* study, Citric Acid has revealed antibiofilm potential against Dual bacterial species (Bacteria + Bacteria). Consequently, Citric Acid could be used as an effective antibiofilm agent to treat microbial biofilms related menaces be it foodborne diseases or nosocomial infections.

CONCLUSION

In the present *in vitro* study, Citric Acid was evaluated for its Antibiofilm potential against total 12 dual bacterial biofilms of *E. coli*, *S. enterica* and *S. typhi* with different Bacteria using crystal violet staining method. Citric Acid exerted antibiofilm effect against dual bacterial biofilms in the following order, *E. coli* + *S. enterica* (91.15%) > *E. coli* + *E. faecalis* > *E. coli* + *MRSA* > *E. coli* + *S. typhi* > *E. coli* + *P. aeruginosa* > *S. enterica* + *MRSA* > *S. typhi* + *MRSA* > *S. typhi* + *E. faecalis* > *S. enterica* + *E. faecalis* > *S. enterica* + *S. typhi* > *S. enterica* + *P. aeruginosa* > *S. typhi* + *P. aeruginosa* (50.58%). Additionally, combination prepared from Citric Acid (CA) and Ciprofloxacin (CP)-standard antibiotic was also evaluated to determine its combinatorial effect against these 12 Dual bacterial biofilms. The combination CA+CP displayed the highest antibiofilm activity against the dual bacterial biofilms of *E. coli* + *S. enterica*, whereas, the lowest antibiofilm effect was noted against the dual bacterial biofilms of *S. typhi* + *P. aeruginosa.* In our previous studies, Citric Acid has displayed antibiofilm potential against Mono and Mixed microbial species, whereas, in current *in vitro* study, Citric Acid exhibited antibiofilm effect against Dual bacterial biofilms. Hence, Citric Acid could be a good antibiofilm agent with efficient, eco-friendly and safe therapeutic application for microbial biofilm-related menaces.

ACKNOWLEDGEMENT

1) Mr. Pavankumar Todkar, Head, Virology Department, Haffkine Institute, Mumbai kindly allowed to use Multimode Reader of Virology Department for the said study.
2) Sub-culturing of bacteria included in the said study and the media preparation for it was carried out by Mrs. Dipti Kolte, Senior Technical Assistant (STA), Bacteriology Department, Haffkine Institute, Mumbai.

REFERENCES

1. Hemeg HA. Nanomaterials for alternative antibacterial therapy. Int J Nanomed, 2017; 12: 8211-8225.
2. Abebe GM. The role of bacterial biofilm in antibiotic resistance and food contamination. Int J Microbiol, 2020; Article ID: 1705814.
3. Hussaini IM, Oyewole OA, Sulaiman MA, Dabban AI, Sulaiman AN, Tarek R. Microbial antibiofilms: Types and mechanism of action. Res Microbiol, 2024; 175: 104111.
4. Devi S, Sharma P, Sharma N, Chauhan S, Sharma A, Thakur M. Microbial biofilms: Beneficial and detrimental impacts. J Microbiol Biotech Food Sci., 2023; 12(5): e5211.
5. Lin Z, Wang G, Li S, Zhou L, Yang H. Dual-species biofilms fprmed by *Escherichia coli* and *Salmonella* enhance chlorine tolerance. Appl Environ Microbiol, 2022; 88: e01482-22.
6. Ruhal R, Kataria R. Biofilm patterns in gram positive and gram negative bacteria. Microbiol Res., 2021; 251: 126829.
7. Książek E. Citric Acid: Properties, Microbial Production, and Applications in Industries. Molecules, 2024; 29: 22.
8. Lambros M, Tran T, Fei Q, Nicolaou M. Citric Acid: A Multifunctional Pharmaceutical Excipient. Pharmaceutics, 2022; 14: 972.
9. Rege AA, Kolte DB, Naik MA. *In vitro* Evaluation of Antimicrobial and Antibiofilm Potential of Citric Acid. World J Pharm Pharm Sci., 2025; 14(7): 1502-1522.
10. Gong C. The role of microbial biofilm in food processing and the importance of foodborne disease surveillance. J Food Microbiol, 2025; 9(1): 246.
11. Yang L, Yang L, Wu H, Hϕiby N, Molin S, Song Z. Current understanding of multi-species biofilms. Int J Oral Sci., 2011; 3: 74-81.
12. Azeredo J, Azevedo NF, Briandet R, Cerca N, Coenye T, Costa AR, Desvaux M, Bonaventura GD, Hébraud M, Jaglic Z, Kačániová M, Knøchel S, Lourenço A, Mergulhão F, Meyer RL, Nychas G, Simões M, Tresse O, Sternberg C. Critical review on biofilm method. Crit Rev Microbiol, 2017; 43(3): 313-351.
13. Pantanella F, Valenti P, Natalizi T, Passeri D, Berlutti F. Analytical techniques to study microbial biofilm on abiotic surfaces: pros and cons of the main techniques currently in use. Ann Ig, 2013; 25: 31-42.
14. O'Toole GA. Microtiter dish biofilm formation assay. J Vis Exp, 2011; JOVE 47. (doi: 10.3791/2437).
15. Gilbert-Girard S, Savijoki K, Yli-Kauhaluoma J, Fallarero A. optimization of a high-throughput 384-well plate-based screening platform with *Staphylococcus aureus* ATCC 25923 and *Pseudomonas aeruginosa* ATCC 15442 biofilms. Int J Mol Sci., 2020; 21: 3034.
16. Shukla SK, Rao TS. An improved crystal violet assay for biofilm quantification in 96-well microtiter plate. 2017; bio Rxiv preprint doi: https//doi.org/10.1101/100214.
17. Sung K, Khan S, Ahn J. Foodborne pathogen biofilms: Development, detection, control and antimicrobial resistance. Pathogens, 2023: 12: 352.
18. Vaou N, Stavropoulou E, Voidarou C, Christina T, Bezirtzoglou E. Towards advances in Medicinal Plant Antimicrobial activity: A review study on challenges and future prospectives. Microorganisms, 2021; 9: 2041.
19. Li Q, Liu L, Guo A, Zhang X, Liu W, Ruan Y. Formation of multispecies biofilm and their resistance to disinfectants in food processing environment: A review. J Food Prot., 2021; 84(12): 2071-2083.
20. Rege AA, Naik MA. *In vitro* Antibiofilm Effect of Citric Acid against Dual and Mixed culture biofilms of C*andida albicans* with different Bacteria. World J Pharm Res., 2025; 14(14): 1310-1324.