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IN VITRO ANTIBACTERIAL AND ANTIBIOFILM ACTIVITIES OF ASCORBIC ACID AGAINST CLINICAL ISOLATES

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ABSTRACT

Infections with MDR and XDR gram negative bacteria pose a significant challenge to clinicians due to severely limited therapeutic options. Besides, health-related concerns are increasing due to the potential of biofilms to cause disorders by both device-related and non-device related bacterial infections. Ascorbic acid could be a possible source of effective, cheap and safe candidate for eradication of biofilms. Hence, in the present *in vitro* study, Antibacterial and Antibiofilm potential of Ascorbic acid was evaluated against 6 Clinical Isolates, namely, *Klebsiella aerogenes*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Proteus vulgaris* and *Shigella flexneri* as well as their Mixed Culture using various assays such as MIC, MBC, Agar Well Diffusion, Time Course study and Antibiofilm assay. The lowest MIC with Ascorbic Acid was noted against *P. aeruginosa*. The Antibiofilm activity was noted in the order of *P. aeruginosa* > *P. mirabilis* > Mixed Culture > *K. pneumoniae* > *K. aerogenes* > *S. flexneri*. Moreover, combination of Ascorbic acid + Ciprofloxacin revealed synergistic effect against Biofilms of *K. aerogenes*, *K. pneumoniae* and *S. flexneri*. Furthermore, in Time Course Study, Ascorbic Acid inhibited all Clinical Isolates and their Mixed Culture within 3 hours. Our previous study has revealed broad spectrum Antibacterial and Antibiofilm effects of Ascorbic acid against Standard Strains inhibiting gram positive as well as gram negative

bacteria and their mixed cultures, whereas as, current *in vitro* study has exhibited Antibacterial and Antibiofilm activities against Clinical Isolates and their mixed culture. Hence, Ascorbic Acid could be a good candidate for further investigations.

KEYWORDS: Ascorbic Acid, Antibacterial, Antibiofilm, Natural Products, Biofilm Assay, Mixed Culture.

INTRODUCTION

Bacterial infections are among prominent causes of health problems, physical disabilities and mortality around the world. In recent decades, the proliferation of bacteria resistant to multiple antibiotics has become increasingly widespread. This trend has posed challenges to effectively managing and treating certain human infectious diseases.[1] In India, infections with MDR and XDR gram negative bacteria are frequent and pose a significant challenge to clinicians due to severely limited therapeutic options.[2] Besides, biofilms are complex microbial communities formed by one or more organisms embedded in an extracellular polymeric matrix produced by them. Biofilm bacteria are more resistant to external factors such as drying, UV radiation and changes in pH and temperature. But they are also less sensitive to disinfectants, antibiotics and mechanisms of innate and acquired immunity.[3] Additionally, biofilms are responsible for majority of chronic infections. Chronic infections are substantial burden to patients and they present a serious economic burden to healthcare systems.[4] Furthermore, treatment of poly-microbial biofilms require antimicrobials that are effective against all microorganisms in the biofilms which creates an additional challenge.[5] Natural compounds are regarded as safe and biodegradable and may penetrate the biofilm structure killing the bacteria. Application of Ascorbic acid is one such alternatives. Ascorbic acid is safe, cheap and easily accessible.[6] Therefore, in the present *in vitro* study, Ascorbic acid was evaluated for its Antibacterial and Antibiofilm potential against 6 Clinical Isolates and their Mixed Culture using various assays such as MIC, MBC, Agar Well Diffusion, Time Course study and Antibiofilm assay.

MATERIALS AND METHODS

1. Preparation of Test Solution

The test solution was prepared by dissolving Ascorbic Acid (SRL) in sterile distilled water (Stock solution= 100 mg/ml) and it was stored at 4^0C until further use. Ascorbic Acid was kindly provided by Mrs. Suvarna Pachpore, Head, Chemical Testing Department, Haffkine Institute, Mumbai.

2. Test Organisms used for Antibacterial Assays

Antibacterial activity of Ascorbic acid was evaluated against 6 Clinical Isolates (Gram Negative Bacteria), namely, *Klebsiella aerogenes*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Proteus mirabilis, Proteus vulgaris* and *Shigella flexneri* as well as their Mixed Culture. Bacterial cultures were grown on Nutrient agar and suspended in Mueller Hinton Broth (MHB) for the assays.

3. Assessment of Minimum Inhibitory Concentration (MIC)

Assessment of Minimum Inhibitory Concentration (MIC) of Ascorbic Acid was carried out according to Rege AA *et al*.[7] with slight modification in Eppendorf tubes. MIC was defined as the lowest concentration of the test solution that restricted the visible growth of microorganism tested. Besides, effect of Ascorbic acid was also assessed against mixed culture, for which the mixed bacterial culture was prepared in 1:1 ratio. Sterile distilled water was used as a negative control, whereas, standard antibiotic such as Ciprofloxacin was used as a positive control.

4. Assessment of Minimum Bactericidal Concentration (MBC)

Assessment of Minimum Bactericidal Concentration (MBC) of Ascorbic Acid was carried out according to Rege AA *et al*.[7] with slight modification. MBC was defined as the lowest concentration of the test solution showing no bacterial growth. Sterile distilled water was used as a negative control, whereas, standard antibiotic such as Ciprofloxacin was used as a positive control.

5. Evaluation of Antibacterial Activity by Agar Well Diffusion Method

Antibacterial activity of Ascorbic acid was performed using the Agar-well diffusion assay according to Rege AA *et al*.[7] with slight modification. Briefly, fresh culture (10^6 CFU/ml) was uniformly spread onto Mueller-Hinton agar (MHA) plates using sterile loop. Then, inoculated plates were allowed to dry at room temperature for 20 min. After that, wells of 6mm in diameter were made in the agar using a sterilized cup-borer and 100 μl of Ascorbic acid solution in different concentrations (10, 50 & 100 mg/ml) was added in the wells. Sterile distilled water was used as a Negative control. Plates were incubated at 37^0C for 24 h. Ciprofloxacin was used as a positive control. Antibacterial activity was evidenced by the presence of clear inhibition zone around each well. The diameter of this zone was measured and recorded. Experimental results were expressed as Mean ± Standard Deviation (SD) for analysis performed in duplicate.

6. Anti-Biofilm Assay

The effect of Ascorbic acid on bacterial biofilm formation was evaluated against 6 Clinical Isolates & their Mixed Culture in sterile 96-well polystyrene flat-bottom microplates according to Rege AA *et al.*[7] with slight modification. Briefly, 200 μl of inoculated fresh Mueller Hinton Broth (10^6 CFU/ml) was aliquoted in triplicate to respective wells of sterile microplate and cultured in presence of test concentrations (0.1, 1 & 10 mg/ml). Wells containing bacterial cultures with distilled water were used as controls. Ciprofloxacin was included as a standard antibiotic. Plates were sealed and 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 during 15 min at room temperature with 0.1% aqueous solution of crystal violet. Following incubation, the excess of stain was removed by washing the plate three times with sterile distilled water. Finally, the dye bound to the cells was 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 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 Ascorbic acid or Ciprofloxacin (standard) respectively. Experimental results were expressed as Mean for analysis performed in triplicate.

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

7. Effect of Before-Treatment and After-Treatment on Bacterial Biofilms

(a) For Before-treatment study, 100 µl of bacterial cultures (10^6 CFU/ml) were added to the sterile microplate in triplicate and the plate was sealed and then incubated at 37^0C for 48 h. After incubation, the cultures were aspirated carefully and 100 µl of Ascorbic acid (10 mg/ml) was added to the plate and the sealed plate was incubated further at 37^0C for 48 h. After incubation, supernatant was removed and the effect of test solutions on bacterial biofilms was determined by Crystal Violet staining method as stated earlier. Ciprofloxacin (2 mg/ml) was included as a standard.

(b) For After-treatment study, 100 µl of Ascorbic acid (10 mg/ml) was added to the sterile microplate in triplicate and the plate was sealed and then incubated at 37^0C for 48 h. After incubation, the test solutions were aspirated carefully and 100 µl of bacterial cultures (10^6 CFU/ml) were added to the microplate and the sealed plate was further incubated at 37^0C for 48 h. After incubation, supernatant was removed and the effect of test solutions on bacterial biofilms was determined by Crystal Violet staining method as stated earlier. Ciprofloxacin (2 mg/ml) was included as a standard.

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

8. Combinatorial Effect on Bacterial Biofilms

In the present study, combinatorial effect of Ascorbic acid (10 mg/ml) and Ciprofloxacin-standard Antibiotic (2 mg/ml) was evaluated using Crystal Violet staining method as stated earlier. Combination was prepared in 1:1 ratio and its effect was tested against 6 bacterial biofilms and biofilm of Mixed Culture.

For the said study, 100 µl of bacterial cultures (10^6 CFU/ml) and 100 µl of combination solution were added to the sterile microplate in triplicate and the plate was sealed and then incubated at 37^0C for 48 h. After incubation, supernatant was removed and the effect of combination solution on bacterial biofilms was determined by Crystal Violet staining method as stated earlier.

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

9. Time Course Study

In the Time Course study, 500 μl of Ascorbic acid (10 mg/ml) solution was transferred to different Eppendorf tubes. Then, 100 μl of a fresh cultures (10^6 CFU/ml) and the Mixed culture (1:1) were added to Eppendorf tubes. The tubes were incubated at different time intervals such as 5min, 3hr, 6hr, 24hr, 27hr, 30hr and 48hr. At each time point, 10 μl from each of the tubes was spotted on Mueller Hinton Agar (MHA) plates and then the plates were incubated at 37^0C for 24 h. The result was noted as presence or absence of growth. Ciprofloxacin (2 mg/ml) was included as a standard.

RESULTS

Table 1: MIC of Ascorbic Acid against Clinical Isolates.

No.	Clinical Isolates	MIC (mg/ml)
1	*K. aerogenes*	6
2	*K. pneumoniae*	6
3	*P. aeruginosa*	2
4	*P. mirabilis*	6
5	*P. vulgaris*	6
6	*S. flexneri*	4
7	Mixed Culture	4

Table 2: Antibacterial Activity of Ascorbic Acid by Agar Well Diffusion Method.

No.	Clinical Isolates	Zone of Inhibition (mm) [Mean ± SD]		
		10 mg/ml	50 mg/ml	100 mg/ml
1	*K. aerogenes*	Nil	18 ± 0	21 ± 1.4
2	*K. pneumoniae*	Nil	16 ± 0	22 ± 0
3	*P. aeruginosa*	Nil	18 ± 0	23 ± 0
4	*P. mirabilis*	Nil	16 ± 0	22.5 ± 0.7
5	*P. vulgaris*	Nil	18 ± 0	22 ± 0
6	*S. flexneri*	Nil	19.5 ± 0	24 ± 0
7	Mixed Culture	Nil	19 ± 0	24 ± 0

Table 3: Effect of Ascorbic Acid on Bacterial Biofilms.

No.	Clinical Isolates	Inhibition (%)		
		0.1 mg/ml	1 mg/ml	10 mg/ml
1	*K. aerogenes*	Nil	Nil	55.26
2	*K. pneumoniae*	Nil	Nil	60.95
3	*P. aeruginosa*	Nil	Nil	76.51
4	*P. mirabilis*	6.96	18.35	75.95
5	*P. vulgaris*	Nil	Nil	Nil
6	*S. flexneri*	Nil	Nil	50.90
7	Mixed Culture	Nil	0.22	71.18

Note: Mean of triplicate determinations

Table 4: Effect of Ciprofloxacin (Standard) on Bacterial Biofilms.

No.	Clinical Isolates	Inhibition (%)
1	*K. aerogenes*	62.94
2	*K. pneumoniae*	56.04
3	*P. aeruginosa*	83.52
4	*P. mirabilis*	68.14
5	*P. vulgaris*	78.12
6	*S. flexneri*	44.40
7	Mixed Culture	67.31

Note: Mean of triplicate determinations

Table 5: Effect of Ascorbic Acid on Bacterial Biofilms.

(Before and After Treatment)

No	Clinical Isolates	Inhibition (%)	
		Before Treatment	After Treatment
1	*K. aerogenes*	Nil	Nil
2	*K. pneumoniae*	Nil	8.93
3	*P. aeruginosa*	Nil	Nil
4	*P. mirabilis*	Nil	Nil
5	*P. vulgaris*	Nil	81.48
6	*S. flexneri*	Nil	40.51
7	Mixed Culture	Nil	33.22

Note: Mean of triplicate determinations

Table 6: Effect of Ciprofloxacin on Bacterial Biofilms.

(Before and After Treatment)

No.	Clinical Isolates	Inhibition (%)	
		Before Treatment	After Treatment
1	*K. aerogenes*	Nil	64.67
2	*K. pneumoniae*	Nil	57.52
3	*P. aeruginosa*	Nil	17.87
4	*P. mirabilis*	7.97	25.17
5	*P. vulgaris*	Nil	54.50
6	*S. flexneri*	32.70	36.86
7	Mixed Culture	Nil	57.55

Note: Mean of triplicate determinations

Table 7: Combinatorial Effect on Bacterial Biofilms.

No.	Clinical Isolates	Inhibition (%) (AA + CP)
1	*K. aerogenes*	75.89
2	*K. pneumoniae*	63.97
3	*P. aeruginosa*	63.09
4	*P. mirabilis*	72.06
5	*P. vulgaris*	59.12
6	*S. flexneri*	63.46
7	Mixed Culture	69.05

Note: Mean of triplicate determinations

AA- Ascorbic Acid

CP- Ciprofloxacin (Standard Antibiotic)

Table 8: Time Course Study with Ascorbic Acid.

No.	Clinical Isolates	Time Points						
		5 min	3 hr.	6 hr.	24 hr.	27 hr.	30 hr.	48 hr.
1	*K. aerogenes*	+	-	-	-	-	-	-
2	*K. pneumoniae*	+	-	-	-	-	-	-
3	*P. aeruginosa*	+	-	-	-	-	-	-
4	*P. mirabilis*	+	-	-	-	-	-	-
5	*P. vulgaris*	+	-	-	-	-	-	-
6	*S. flexneri*	+	-	-	-	-	-	-
7	Mixed Culture	+	-	-	-	-	-	-

DISCUSSION

According to the National Institute of Health (NIH), up to 80% of human bacterial infections are caused by bacterial biofilms which are extremely hard to cure. Health-related concerns are increasing due to the potential of biofilms to cause disorders by both device-related and non-device related bacterial infections. The biofilm-associated bacterial infection is considered as a significant clinical problem. The biofilm structures can compromise the human defences and serve as a shelter for microorganisms, leading to immune system evasion, bacterial persistence, drug tolerance and resistance. Therefore, prevention of bacterial biofilm-associated infections is highly needed.[8] Ascorbic acid could be a possible source of effective, cheap and safe candidate for eradication of biofilms. Hence, in the present *in vitro* study, antibacterial and antibiofilm activities of Ascorbic acid were evaluated against 6 Clinical Isolates (Gram negative bacteria) and their Mixed Culture using various assays such as MIC, MBC, Agar Well Diffusion Method, Time Course study and Antibiofilm assay.

Conventional Antimicrobial Susceptibility Testing based on phenotypic testing examines the bacterial response in the presence of an antimicrobial agent. By using broth and agar dilution methods, the minimum inhibitory concentration (MIC) of antimicrobial agents (i.e., the lowest concentration at which the agent inhibits the growth of microorganisms) can be determined. Broth dilution uses, serial dilution of antimicrobial substances in corresponding media. After 24 h of incubation at 37^0C, bacterial growth is measured by turbidity of media, allowing visual determination of MIC values.[9]

In the present *in vitro* study, in general, the lowest MIC with Ascorbic Acid was noted against *P. aeruginosa*, whereas, highest MIC was found to be at a concentration of 6 mg/ml against *K. aerogenes*, *K. pneumoniae*, *P. mirabilis* and *P. vulgaris* [Table-1].

Likewise, Ciprofloxacin a standard antibiotic showed MIC of more than 2 mg/ml against *K. aerogenes*, *K. pneumoniae*, *P. mirabilis*, *S. flexneri* and Mixed culture, whereas, it showed MIC of less than 0.1 mg/ml against *P. aeruginosa* and *P. vulgaris*.

Minimum Bactericidal Concentration (MBC) can be determined after broth dilution by using agar plates to confirm the antimicrobial potential of tested natural products.[10] Accordingly, the observations of MIC were confirmed by Minimum Bactericidal Concentration (MBC) assay using Muller Hinton Agar (MHA) plates [photos not included]. It is clear from the result that the inhibitory activity of Ascorbic acid was found to be concentration dependent (as the concentration increased, growth decreased).

Besides, Agar well diffusion method is widely used to evaluate the antimicrobial activity of natural products, wherein the agar plate surface is inoculated by spreading a volume of microbial culture over the entire agar surface. Then a hole with a diameter of 6 to 8 mm is punched aseptically with a sterile cork borer and a volume of antimicrobial agent at desired concentration is introduced into the well. Then, the agar plates are incubated under suitable conditions depending upon the test microorganisms. The antimicrobial agent diffuses in the agar medium and inhibits the growth of microbial strain tested.[10]

In the present study, three different concentrations (10, 50 and 100 mg/ml) of Ascorbic Acid were evaluated for their antibacterial effect using Agar Well Diffusion method against 6 bacteria and their mixed culture. In general, the highest zone of inhibition was noted at a concentration of 100 mg/ml against *S. flexneri* and Mixed Culture and the inhibitory activity was found to be concentration dependent, viz., inhibitory effect increased with increasing concentration. Ascorbic Acid revealed the inhibitory activity in the order of *S. flexneri* = Mixed culture > *P. aeruginosa* > *P. mirabilis* > *K. pneumoniae* = *P. vulgaris* > *K. aerogenes*. Furthermore, no zones of inhibition were noted against any of the organisms at a concentration of 10 mg/ml [Table-2].

The standard antibiotic Ciprofloxacin which was included as a positive control in the present study displayed the highest zone of inhibition against *P. aeruginosa* and the inhibitory activity was further noted in the order of *P. aeruginosa* > *P. vulgaris* > *S. flexneri* > *K. aerogenes* = *K. pneumoniae* = *P. mirabilis* > Mixed culture at the concentration of 2 mg/ml.

Overall, Ascorbic Acid exhibited strong Antibacterial activity against the Clinical Isolates included in the present study along with their mixed culture which is revealed through MIC, MBC and Agar well Diffusion Assays. Hence, potential of Ascorbic Acid was further evaluated against bacterial biofilms using crystal violet staining method.

Biofilms are the predominant growth state of many microorganisms.[11] Biofilms have great importance for public health because of their role in certain infectious diseases and involvement in variety of device-related infections.[12] Besides, chronic infections are frequently caused by polymicrobial (mixed species) biofilms and these infections are often difficult to treat effectively due to the recalcitrance of biofilms to antimicrobial therapy.[13] Hence, Anti-Biofilm potential of Ascorbic acid was evaluated against 6 Clinical Isolates and their Mixed culture 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. 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.[14]

Likewise, the microtiter plate assay is an important tool for the study of the early stages in biofilm formation and has been applied primarily for the study of 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.[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 significantly 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. To assess the capability of test solutions to prevent the growth of biofilms, the

bacterial cultures were incubated in presence of test solutions. Three concentrations of Ascorbic acid mainly, 0.1 mg/ml, 1 mg/ml and 10 mg/ml were included in the Anti-biofilm assay in the present study. In general, higher inhibition with Ascorbic Acid was noted at the concentration of 10 mg/ml against bacteria. The Antibiofilm activity was further noted in the order of *P. aeruginosa* > *P. mirabilis* > Mixed Culture > *K. pneumoniae* > *K. aerogenes* > *S. flexneri.* However, it did not show any Anti-biofilm activity against *P. vulgaris* at any concentrations tested. Additionally, in case of *P. mirabilis* the Antibiofilm activity of Ascorbic Acid was observed at all the 3 concentrations and the activity was found to be concentration dependent, i.e., inhibition increased with the increasing concentration. However, no antibiofilm effect was noted against *K. aerogenes, K. pneumoniae, P. aeruginosa* and *S. flexneri* at concentrations of 0.1 mg/ml and at 1 mg/ml [Table-3].

The standard antibiotic- Ciprofloxacin which was included as a positive control in the present study, revealed the highest Anti-Biofilm activity against *P. aeruginosa* (83.52%) at the concentration of 2 mg/ml followed by *P. vulgaris* > *P. mirabilis* > Mixed culture > *K. aerogenes* > *K. pneumoniae* > *S. flexneri* [Table-4].

Antibiofilm assay was further divided into two separate and additional parts, viz., Before-treatment and After-treatment with Ascorbic acid individually. In Before-treatment assay, the ability of test solutions to eradicate already established biofilms was evaluated, for which the bacterial cultures were incubated first in the microplate wells for 48 h at 37^0C. Then the cultures were replaced with Ascorbic acid and the plates were further incubated for 48 h at 37^0C.

Besides, preconditioning of the surfaces with antimicrobial agents renders unfavourable conditions for the initial stage (attachment) of biofilm formation.[17] Hence, in After-treatment assay, the microplate wells were first incubated with Ascorbic acid for 48 h at 37^0C. Then the test solutions were replaced with bacterial cultures and the plates were further incubated for 48 h at 37^0C. Ascorbic Acid exhibited inhibitory activity in After-Treatment assay only mainly against *P. vulgaris, S. flexneri,* Mixed Culture and *K. pneumoniae*. However, it did not show any inhibitory effect against *K. aerogenes, P. aeruginosa* and *P. mirabilis* in Before as well as After Treatment assays [Table-5].

The standard antibiotic Ciprofloxacin showed inhibitory effect against *P. mirabilis* and *S. flexneri* only in Before-treatment assay. While, in After-treatment assay, the highest

inhibitory activity by Ciprofloxacin was observed against *K. aerogenes* followed by Mixed Culture > *K. pneumoniae* > *P. vulgaris* > *S. flexneri* > *P. mirabilis* > *P. aeruginosa* [Table-6].

Combining antibiofilm agents with antibiotics is emerging as a promising strategy to eradicate biofilms.[18] Hence, combination prepared from Ascorbic Acid (AA) and Ciprofloxacin (CP)- standard antibiotic was evaluated to determine its combinatorial effect against biofilms of 6 Clinical Isolates and their Mixed culture using crystal violet staining method. In general, the combination AA + CP displayed the highest Anti-biofilm activity against *K. aerogenes* and the inhibitory activity was further noted in the order of *K. aerogenes* > *P. mirabilis* > Mixed Culture > *K. pneumoniae* > *S. flexneri* > *P. aeruginosa* > *P. vulgaris* [Table- 7].

In general, combination AA + CP revealed synergistic effect against Biofilms of *K. aerogenes, K. pneumoniae and S. flexneri*. The combination displayed increased inhibitory activity than that of Ascorbic acid and Ciprofloxacin individually.

The synergistic effect of Ascorbic acid with antibiotics could be due to its effect on certain metabolic activities associated with protein synthesis inside bacterial cells making the bacterial cells more permeable to antibiotics through its effect on the cytoplasmic membrane or it could be due to the effect of hydrogen peroxide produced by the oxidation of Ascorbic acid which causes antibiotics to have a higher potency. Also, the synergistic effect of Ascorbic acid with antibiotics could be due to down regulation of antibiotic-resistant genes.[19]

Besides, in the present *in vitro* study, Ascorbic acid was subjected to Time Course study to estimate the time point at which the organisms are inhibited by it. For this, 6 Clinical Isolates along with their Mixed culture were incubated with test solution for different time points such as 5min, 3hr, 6hr, 24hr, 27hr, 30hr and 48hr.

The Time Course Study with Ascorbic Acid (10 mg/ml) revealed inhibition of all Clinical Isolates and their Mixed Culture within 3 hours [Table-8].

The standard antibiotic Ciprofloxacin displayed inhibition of *P. aeruginosa* and *P. vulgaris* within 5 min. However, it did not inhibit rest of bacteria and their Mixed Culture even at 48 hr. This observation correlates with MIC result of Ciprofloxacin.

Additionally, earlier study revealed antioxidant potential of Ascorbic Acid which was detected using DPPH assay.[20] The presence of both antioxidant and antimicrobial properties in a single molecule makes them more effective.[21]

One of the main mechanisms that drives a microorganism to transit from a planktonic to a biofilm-sessile state is oxidative stress. Oxidative stress encountered by the bacterial cells could be caused by abiotic stresses, presence of antimicrobials or the host immune system. Moreover, an elevation of reactive oxygen species (ROS) has been reported to upregulate certain microbial proteins which in turn leads to biofilm formation. Thus, oxidative stress in microorganisms does play an important role in the regulation of redox-defence mechanisms, the production of EPS and biofilm heterogeneity. Chemical compounds that could target oxidative stress for instance antioxidants like Ascorbic Acid could therefore be used to treat biofilm-associated infections. Antioxidants were shown to possess potent anti-biofilm properties as they can reduce oxidative stress-mediated virulence in pathogenic microorganisms by scavenging free radicals.[22]

In general, Ascorbic Acid has revealed Antibacterial and Antibiofilm potential against mono as well as mixed culture in present *in vitro* study. Its mechanism of action could not only be anti-quorum sensing activity and inhibition of production of extracellular polymeric substances, but also its ability to lower the pH in the environment, providing unsuitable conditions for bacteria to survive.[23,24] Besides, our previous study has revealed broad spectrum Antibacterial and Antibiofilm effects of Ascorbic acid inhibiting gram positive as well as gram negative bacteria.[25]

Furthermore, Ascorbic acid was found to be stable at various temperatures (4^0 C, 37^0 C & 50^0 C) as well as at different pH (acidic & basic) in the study conducted by Mumtaz *et al.*[26] which could be an added advantage to use Ascorbic acid as Antibacterial and Antibiofilm agent.

Thus, Ascorbic Acid epitomizes a possible source of effective, cheap and safe Antibacterial and Antibiofilm agent.

CONCLUSION

In the present *in vitro* study, Antibacterial and Antibiofilm potential of Ascorbic acid was tested against 6 Clinical Isolates (Gram Negative Bacteria) and their Mixed culture using

various assays such as MIC, MBC, Agar Well Diffusion Method, Time Course study and Antibiofilm assay. The current *in vitro* study has exhibited Antibacterial and Antibiofilm potential of Ascorbic acid against Mono as well as Mixed Culture. Moreover, combination of Ascorbic acid + Ciprofloxacin has revealed synergistic effect against *K. aerogenes*, *K. pneumoniae and S. flexneri*. Thus, Ascorbic Acid could be a good candidate for further investigations.

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