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IN VITRO ANTIFUNGAL AND ANTIBIOFILM POTENTIAL OF ASCORBIC ACID AGAINST *CANDIDA ALBICANS* AND ITS MIXED CULTURE WITH BACTERIA

[1]*Anuya Aparna Rege, [2]Dipti Bhushan Kolte and [3]Disha Thakur

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

[2]Senior Technical Assistant (STA), Bacteriology Department, Haffkine Institute for Training, Research and Testing, Parel, Mumbai- 400012, India.

[3]Short Term Research Internship Program (STRIP) Student, Bacteriology Department, Haffkine Institute for Training, Research and Testing, Parel, Mumbai- 400012, India.

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

ABSTRACT

The resistance of *Candida albicans* to antifungal agents has been attributed to formation of biofilms which can occur on mucosa as well as on medical devices. The discovery of effective natural product with antibiofilm activity will substantially impact management of *C. albicans* infections as well as other related bacterial infections. Ascorbic acid could be a possible candidate for eradication of biofilms. Hence, in the present *in vitro* study, antifungal and antibiofilm activities of Ascorbic acid were evaluated against 2 fungi as well as against combination of *C. albicans* with Mixed bacteria (*C. albicans* + Mixed Bacteria) using various assays such as MIC, MBC/MFC, Agar Well Diffusion Method, Time Course study and Antibiofilm assay. Ascorbic Acid did not show any inhibitory effect against *A. niger*. However, Antibiofilm effect of Ascorbic Acid against *C. albicans* and its Mixed Culture was observed at 37^0 C as well as at RT (20^0 C) and it was found to be concentration dependent. Furthermore, combination of Ascorbic Acid + Fluconazole revealed synergistic effect against Biofilms of *C. albicans* and its Mixed Culture. Moreover, the Time Course Study with Ascorbic Acid revealed inhibition of *C. albicans* and its Mixed Culture around 48 hours and 6 hours respectively. In general, Ascorbic Acid has revealed Antifungal and Antibiofilm potential against mono as well as polymicrobial culture

in present *in vitro* study. Besides, our previous studies have revealed broad spectrum Antibacterial and Antibiofilm effects of Ascorbic acid inhibiting Gram Positive and Gram Negative Standard Strains as well as Gram Negative Clinical Isolates. Consequently, Ascorbic Acid exemplifies a promising source of effective, cheap and safe Antimicrobial and Antibiofilm agent.

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

INTRODUCTION

Candida albicans while generally harmless commensal in healthy individuals, several factors can lead to its overgrowth and cause a range of complications within the host from localized superficial infections to systemic life-threatening infections. Besides, a major virulence factor of *C. albicans* is its ability to form biofilms and it can form complex biofilms on medical devices. These biofilms are extremely hard to eradicate, they are resistant to conventional antifungal treatment and are associated with high morbidity and mortality rates, making biofilm-associated infections a major clinical challenge.[1] Recent scientific evidence has shown that *Candida* biofilms constitute a serious economic and health issues in food industry.[2] Furthermore, *C. albicans* and bacteria can interact with each other by secretion of signalling molecules. These interactions occurring between different species in polymicrobial biofilms and their relevance to human health are clearly of great importance.[3] Mixed bacterial-fugal biofilms are associated with infections of catheters, orthopaedic prostheses, endotracheal tubes, acrylic dentures and biliary stents to name a few.[4] Such polymicrobial biofilms are notoriously difficult to eliminate and are a source of many recalcitrant infections.[5] Natural products have shown antibiofilm activity and eradication of *C. albicans* biofilms in the past.[6] Application of Ascorbic acid is one such alternatives. Ascorbic acid is safe, cheap and easily accessible.[7] Therefore, in the present *in vitro* study, Antifungal and Antibiofilm activities of Ascorbic acid were evaluated against 2 fungi. Besides, Ascorbic Acid exhibited strong Antibacterial activity against the clinical isolates (Gram negative bacteria) as well as on their Mixed Culture in our previous study.[8] Therefore, antimicrobial and antibiofilm activities of Ascorbic acid was also evaluated against combination of *C. albicans* with Mixed bacterial Culture (*C. albicans* + Mixed Bacteria) using various assays such as MIC, MBC/MFC, Agar Well Diffusion Method, 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 Antimicrobial Assays

Antifungal activity of Ascorbic acid was evaluated against 2 fungi *Aspergillus niger* (ATCC-16404) *& Candida albicans* (ATCC-10231) and a Mixed Culture (*C. albicans* + Mixed Bacteria). Six Clinical Isolates (Gram Negative Bacteria), namely, *Klebsiella aerogenes*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Proteus vulgaris* and *Shigella flexneri* were included in the mixed bacterial culture. Bacterial cultures were grown on Nutrient agar and suspended in Mueller Hinton Broth (MHB) for the assays, whereas, fungal cultures were grown on Sabouraud Dextrose Agar and were suspended in Sabouraud Dextrose Broth 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*.[9] 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 (*C. albicans* + Mixed Bacteria), for which the mixed culture was prepared in 1:1 ratio. Sterile distilled water was used as a negative control, whereas, standard antibiotics such as Ciprofloxacin and Fluconazole were used as positive control.

4. Assessment of Minimum Bactericidal/Fungicidal Concentration (MBC/MFC)

Assessment of Minimum Bactericidal/Fungicidal Concentration (MBC/MFC) of Ascorbic Acid was carried out according to Rege *et al*.[9] with slight modification. Briefly, to determine MFC, 10 μl from each concentration from MIC tubes was spotted on Sabouraud Dextrose Agar for Fungi and then the plates were incubated at RT (20^0 C) for 48 h.

For Mixed Culture (*C. albicans* + Mixed Bacteria), 10 μl from each concentration from MIC tubes was spotted on Mueller Hinton Agar (MHA) plates and the MHA plates were incubated at 37^0C for 24 h. MBC/MFC was defined as the lowest concentration of the test solution

showing no bacterial/fungal growth. Sterile distilled water was used as a negative control, whereas, standard antibiotics such as Ciprofloxacin and Fluconazole were used as positive control.

5. Evaluation of Antimicrobial Activity by Agar Well Diffusion Method

Antimicrobial activity of Ascorbic acid was performed using the Agar-well diffusion assay according to Rege *et al*.[9] with slight modification. Briefly, fresh fungal culture (10^6 CFU/ml) was uniformly spread onto Sabouraud Dextrose agar 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. Fluconazole was used as positive control/Standard. Plates were incubated at RT (20^0 C) for 48 h for Fungi.

For Mixed Culture (*C. albicans* + Mixed Bacteria), the Mueller-Hinton agar (MHA) plates were used and one set of the plates was incubated at 37^0C for 24 h, whereas, other set was incubated at RT (20^0 C) for 48 h as the mixture contains both bacteria and fungi. Ciprofloxacin and Fluconazole were used as positive control/Standards. Antimicrobial 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 microbial biofilm formation was evaluated in sterile 96-well polystyrene flat-bottom microplates according to Rege *et al*.[9] 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. For *C. albicans* and Mixed Culture (*C. albicans* + Mixed Bacteria), the concentrations such as 1, 10, 50 & 100 mg/ml were used and one set of microplates was incubated at 37^0C for 48h, whereas, other set of microplates was incubated at RT for 48h respectively. Wells containing microbial cultures with distilled water were used as controls. Ciprofloxacin and Fluconazole were included as a standard antibiotics. 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 microbial 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) or Fluconazole (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 Microbial Biofilms

(a) For Before-treatment study against *C. albicans* and Mixed Culture (*C. albicans* + Mixed Bacteria), 100 µl of microbial culture (10^6 CFU/ml) was added to the microplates in triplicate and the plates were incubated at 37^0C for 48 h. After incubation, the cultures were aspirated carefully and Ascorbic Acid (100 mg/ml) was added to the plates and the plates were incubated further at 37^0C for 48 h. After incubation, supernatant was removed and the effect of Ascorbic Acid on microbial biofilms was determined by Crystal Violet staining method as stated earlier. Ciprofloxacin (2 mg/ml) and Fluconazole (2 mg/ml) were included as standards.

(b) For After-treatment study against *C. albicans* and Mixed Culture (*C. albicans* + Mixed Bacteria), 100 µl of Ascorbic acid (100 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 microbial 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 microbial biofilms was determined by Crystal Violet staining method as stated earlier. Ciprofloxacin (2 mg/ml) and Fluconazole (2 mg/ml) were included as a standards.

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 Microbial Biofilms

a) For *C. albicans*, combination of Ascorbic Acid (100 mg/ml) and Fluconazole (2 mg/ml) were prepared in 1:1 ratio and their combinatorial effect was tested against biofilm of *C. albicans*. For the said study, 100 µl of fungal culture and 100 µl of combination solution were added to the microplates in triplicate. One set of microplates was incubated at 37^0C for 48h, whereas, other set of microplates was incubated at RT (20^0 C) for 48h. After incubation, supernatant was removed and the effect of combination solution on fungal biofilm was determined by Crystal Violet staining method as stated earlier.

b) For Mixed Culture (*C. albicans* + Mixed Bacteria), following Combinations of Ascorbic Acid (100 mg/ml), Ciprofloxacin (2 mg/ml) and Fluconazole (2 mg/ml) were prepared in 1:1 ratio and their combinatorial effect was tested against biofilm of Mixed Culture.

- Ciprofloxacin + Fluconazole (CP + F)
- Ascorbic Acid + Ciprofloxacin (AA + CP)
- Ascorbic Acid + Fluconazole (AA + F)
- Ascorbic Acid + Ciprofloxacin + Fluconazole (AA + CP + F)

For the said study, 100 µl of microbial culture and 100 µl of combination solution were added to the microplates in triplicate. One set of microplates was incubated at 37^0C for 48h, whereas, other set of microplates was incubated at RT (20^0 C) for 48h. After incubation, supernatant was removed and the effect of combination solution on microbial biofilm 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 (100 mg/ml) solution was transferred to different Eppendorf tubes. Then, 100 µl of *C. albicans* culture (10^6 CFU/ml) and its Mixed culture (1:1) were added to separate Eppendorf tubes. The tubes were incubated at 37^0C for Mixed Culture, whereas, tubes were incubated at RT (20^0 C) for *C. albicans* 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 for Mixed Culture and on Sabouraud Dextrose Agar for *C. albicans*. The plates were then incubated at 37^0C for 24 h

for Mixed Culture and at RT (20^0 C) for 48 h for *C. albicans*. The result was noted as presence or absence of growth. Ciprofloxacin (2 mg/ml) and Fluconazole (2 mg/ml) were included as standards.

RESULTS

Table 1: MIC of Ascorbic Acid.

No.	Microbes	MIC (mg/ml)
1	*A. niger* (ATCC-16404)	>100
2	*C. albicans* (ATCC-10231)	100
3	Mixed Culture (*C. albicans* + Mixed Bacteria)	100

Table 2: MIC of Standard Antibiotics.

No.	Microbes	MIC (mg/ml)	
		Fluconazole	Ciprofloxacin
1	*A. niger* (ATCC-16404)	2	-
2	*C. albicans* (ATCC-10231)	0.4	-
3	Mixed Culture (*C. albicans* + Mixed Bacteria)	>2	>2

Table 3: Antifungal Activity of Ascorbic Acid by Agar Well Diffusion Method.

No.	Fungi	Zone of Inhibition (mm) [Mean ± SD]		
		10 mg/ml	50 mg/ml	100 mg/ml
1	*A. niger* (ATCC-16404)	Nil	Nil	Nil
2	*C. albicans* (ATCC-10231)	Nil	Nil	Nil

Table 4: Antimicrobial Activity of Ascorbic Acid against Mixed Culture (*C. albicans* + Mixed Bacteria) by Agar Well Diffusion Method.

Concentration (mg/ml)	Zone of Inhibition (mm) [Mean ± SD]	
	at 37^0C for 24 hr.	at RT for 48 hr.
10	Nil	Nil
50	18 ± 0	17 ± 0
100	22 ± 0	22 ± 0

Table 5: Effect on *C. albicans* Biofilm.

Test	Concentration (mg/ml)	Inhibition (%)	
		At 37^0 C	at RT (20^0 C)
Ascorbic Acid (AA)	1	3.96	26.33
	10	59.50	67.37
	50	75.07	73.42
	100	75.46	78.99
Fluconazole (F)	2	1.98	Nil

Note: Mean of triplicate determinations

Table 6: Effect on Biofilm of Mixed Culture (*C. albicans* + Mixed Bacteria)

Test	Concentration (mg/ml)	Inhibition (%)	
		at 37^0 C	at RT (20^0 C)
Ascorbic Acid (AA)	1	Nil	1.94
	10	53.08	82.09
	50	87.32	89.37
	100	87.47	89.86
Ciprofloxacin (CP)	2	86.41	88.65
Fluconazole (F)	2	47.84	Nil

Note: Mean of triplicate determinations

Table 7: Effect of Ascorbic Acid on *C. albicans* Biofilm (Before and After Treatment).

No.	Test	Inhibition (%)	
		Before Treatment	After Treatment
1	Ascorbic Acid	Nil	Nil
2	Fluconazole	16.60	10.99

Note: Mean of triplicate determinations

Table 8: Effect of Ascorbic Acid on Mixed Culture (*C. albicans* + Mixed Bacteria) Biofilm (Before and After Treatment).

No.	Test	Inhibition (%)	
		Before Treatment	After Treatment
1	Ascorbic Acid	Nil	36.81
2	Ciprofloxacin	Nil	79.74
3	Fluconazole	Nil	12.84

Note: Mean of triplicate determinations

Table 9: Combinatorial Effect on *C. albicans* Biofilm.

No.	Combination	Inhibition (%)	
		at 37^0 C	at RT (20^0 C)
1	AA + F	81.27	77.50

Note: Mean of triplicate determinations

AA- Ascorbic Acid

F- Fluconazole (Standard Antibiotic)

Table 10: Combinatorial Effect on Mixed Culture (*C. albicans* + Mixed Bacteria) Biofilm.

No.	Combination	Inhibition (%)	
		at 37^0 C	at RT (20^0 C)
1	CP + F	86.10	91.99
2	AA + CP	87.09	88.59
3	AA + F	87.93	89.74
4	AA + CP + F	87.93	91.01

Note: Mean of triplicate determinations

AA- Ascorbic Acid

CP- Ciprofloxacin (Standard Antibiotic)

F- Fluconazole (Standard Antibiotic)

Table 11: Time Course Study with Ascorbic Acid and Fluconazole against *C. albicans*.

Test (Concentration)	Time Points						
	5 min	3 hr.	6 hr.	24 hr.	27 hr.	30 hr.	48 hr.
Ascorbic Acid (100 mg/ml)	+	+	+	+	+	+	-
Fluconazole (2 mg/ml)	-	-	-	-	-	-	-

Table 12: Time Course Study with Ascorbic Acid, Ciprofloxacin and Fluconazole against Mixed Culture (*C. albicans* + Mixed Bacteria).

Test (Concentration)	Time Points						
	5 min	3 hr.	6 hr.	24 hr.	27 hr.	30 hr.	48 hr.
Ascorbic Acid (100 mg/ml)	+	+	-	-	-	-	-
Ciprofloxacin (2 mg/ml)	+	+	+	+	+	+	+
Fluconazole (2 mg/ml)	+	+	+	+	+	+	+

DISCUSSION

The rapid emergence and spread of antimicrobial resistance has become a global public health concern that threatens the effective treatment of infectious diseases. The resistance of *Candida albicans* to antifungal agents has been attributed to formation of biofilms which can occur on mucosa or endothelial surfaces as well as on medical devices. One major approach adopted to overcome antimicrobial resistance is to use natural products individually and/or with combination of antibiotics. The discovery of effective natural product with antibiofilm activity will signify substantial impact on treatment and management of *C. albicans* infections as well as other related bacterial infections.[10] Ascorbic acid could be a possible source of effective, cheap and safe candidate for eradication of biofilms. Hence, in the present *in vitro* study, antifungal and antibiofilm activities of Ascorbic acid were evaluated against 2 fungi. Besides, Ascorbic Acid exhibited strong Antibacterial activity against the Clinical Isolates (Gram Negative bacteria) as well as on their Mixed Culture in our previous study.[8] Therefore, Antimicrobial and Antibiofilm activities of Ascorbic acid was also evaluated against combination of *C. albicans* with Mixed bacterial Culture using various assays such as MIC, MBC/MFC, Agar Well Diffusion Method, Time Course study and Antibiofilm assay.

The *in vitro* antimicrobial assays play a crucial role in the discovery and development of new antimicrobial agents, providing crucial insights into their effectiveness and mechanism of action.[11] Dilution methods are reference methods for the antimicrobial susceptibility testing (AST) and are used to determine the Minimum Inhibitory Concentration (MIC) values of antimicrobial agents. The dilution methods can be used to quantitatively measure the antimicrobial activity against bacteria, yeasts and filamentous fungi.[12] In the present *in vitro* study, Broth macrodilution method was used to determine MIC values. Broth macrodilution method involves incorporating different concentrations of antimicrobial substance into medium in tubes followed by addition of a standardized inoculum of the test microorganism in the tubes. Subsequently, the tubes are incubated under controlled conditions which is followed by assessment of growth of microorganisms. The MIC is defined as the lowest drug concentration that inhibits visible growth of microorganisms. Thus, the MIC distinguishes susceptible and non-susceptible organisms.[11,12]

In the present *in vitro* study, in general, MIC was noted at the concentration of 100 mg/ml against *C. albicans* and Mixed Culture (*C. albicans* + Mixed Bacteria), whereas, Ascorbic acid showed MIC of more than 100 mg/ml against *A. niger* [Table-1].

Likewise, MIC of Fluconazole was found to be 2 mg/ml and 0.4 mg/ml against *A. niger* and *C. albicans* respectively, whereas, Ciprofloxacin and Fluconazole exhibited MIC of more than 2 mg/ml against Mixed Culture (*C. albicans* + Mixed Bacteria) [Table- 2].

Minimum Bactericidal Concentration (MBC)/ Minimum Fungicidal Concentration (MFC) can be determined after broth dilution by using agar plates to confirm the antimicrobial potential of tested natural products.[13] Accordingly, the observations of MIC were confirmed by Minimum Bactericidal Concentration (MBC) assay using Muller Hinton Agar (MHA) plates for Mixed Culture (*C. albicans* + Mixed Bacteria) and by Minimum Fungicidal Concentration (MFC) assay using Sabouraud Dextrose agar plates for Fungi [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) except against *A. niger* as there was no inhibition even at the highest concentration that was tested.

Besides, Agar diffusion technique has a rich history dating back to the late 18th century, when Beijerinck first utilized it to study auxin's effect on the growth of bacteria. Since then, the method has undergone several modifications to enhance accuracy and efficiency, including

use of wells and filter paper discs. Agar well diffusion assay remains widely utilized, valuable and cost-effective technique used for the preliminary screening of antimicrobial activity in test compounds. This method relies on the diffusion of antimicrobial agents from wells into the adjacent agar medium, inhibiting the growth of test microorganism inoculated on the agar surface. By measuring the resulting zone of inhibition, relative potency of test compound can be assessed against specific organism under investigation.[11]

In the present study, three different concentrations (10, 50 and 100 mg/ml) of Ascorbic Acid were evaluated for their antimicrobial effect using Agar Well Diffusion method against 2 Fungi and the Mixed Culture (*C. albicans* + Mixed Bacteria). In general, no zones of inhibition were observed against Fungi at any concentrations tested [Table-3]. Besides, Agar Well Diffusion assay was carried out at 37^0 C as well as at Room Temperature (RT= 20^0 C) against Mixed Culture as it includes *C. albicans* & Bacteria and similar Zones of inhibition were observed under both the conditions [Table-4]. Furthermore, no zones of inhibition were noted against Mixed Culture at a concentration of 10 mg/ml, whereas, the highest inhibitory activity was observed at the concentration of 100 mg/ml and the inhibitory activity was found to be concentration dependent, viz., inhibitory effect increased with increasing concentration [Table-4]. Usually fast-growing microbial species are said to have more susceptibility to antimicrobial agents than slow growing microorganisms. For example, a fungus requires more time to proliferate and hence, killing requires additional time than the bacterial species. At the same time drug permeability through the fungal membrane are much slower than the bacterial membranes. It should be further noted that different components have different diffusion abilities through the selected medium. These differences may contribute to the strength of antimicrobial action of a particular natural product.[14] This could be the explanation for the moderate activity found against Mixed Culture and no activity noticed against the fungal strains in the Agar Well Diffusion assay.

The standard antibiotic Fluconazole displayed Zone of Inhibition against *C. albicans* only and neither against *A. niger* nor against Mixed Culture (*C. albicans* + Mixed Bacteria). However, the standard antibiotic Ciprofloxacin displayed zone of inhibition (20 mm) against Mixed Culture (*C. albicans* + Mixed Bacteria).

Overall, Ascorbic Acid exhibited strong Antibacterial activity against the clinical isolates (Gram Negative Bacteria) along with their Mixed Culture in our previous study[8], whereas, it exhibited moderate Antifungal activity mainly against *C. albicans* in the current study as

revealed through MIC and MFC Assays. Therefore, effect of Ascorbic Acid was also evaluated against Mixed Culture (*C. albicans* + Mixed Bacteria) and it displayed moderate inhibitory activity against Mixed Culture through MIC, MBC/MFC and Agar well Diffusion Assays. Hence, potential of Ascorbic Acid was further evaluated against biofilm of *C. albicans* and Mixed Culture (*C. albicans* + Mixed Bacteria) using crystal violet staining method.

The National Institute of Health (NIH) has estimated that about 80% of human chronic infections are related to microbial biofilm formation. Biofilm formation by *C. albicans* is implicated in the majority of hospital-acquired and medical-device-associated infections. Furthermore, microbial biofilms are highly heterogeneous in their composition, usually consisting of both bacteria and fungi that are encased within a polysaccharide matrix. Such polymicrobial biofilm infections are more virulent than infections caused by single-species biofilm and are associated with high rates of mortality.[15] Hence, Anti-Biofilm potential of Ascorbic acid was evaluated against *C. albicans* and the Mixed Culture (*C. albicans* + Mixed 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. 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.[16]

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 microbial 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.[17] 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.[18] 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 microbial cultures were incubated in presence of test solutions. Four concentrations of Ascorbic acid mainly, 1 mg/ml, 10 mg/ml, 50 mg/ml and 100 mg/ml were included against *C. albicans* and Mixed Culture (*C. albicans* + Mixed Bacteria) in the Anti-biofilm assay in the present study. Besides, the Antibiofilm assay was carried out at 37^0 C as well as at Room Temperature (RT= 20^0 C) against *C. albicans* and Mixed Culture. In case of *C. albicans*, Antibiofilm effect of Ascorbic Acid was observed at 37^0 C as well as at RT and it was found to be concentration dependent, i.e., inhibition increased with the increasing concentration. Furthermore, the highest Antibiofilm activity was noted at a concentration of 100 mg/ml at both the temperatures [Table-5]. However, antibiofilm activity against *C. albicans* was found to be better at RT. In case of Mixed Culture, Antibiofilm effect of Ascorbic Acid was observed at 37^0 C as well as at RT (20^0 C) and it was found to be concentration dependent, i.e., inhibition increased with the increasing concentration. However, no antibiofilm effect was observed at a concentration of 1 mg/ml at 37^0 C. Furthermore, the highest Antibiofilm activity was noted at a concentration of 100 mg/ml at both the temperatures [Table-6].

The standard antibiotic- Ciprofloxacin which was included as a positive control in the present study, revealed Antibiofilm effect against Mixed Culture at 37^0 C as well as at RT (20^0 C) [Table-6]. Moreover, the standard antibiotic- Fluconazole revealed mild Antibiofilm activity against *C. albicans* and moderate Antibiofilm activity against Mixed Culture that to at 37^0 C only. However, Fluconazole did not show any inhibitory effect at RT against *C. albicans* as well as Mixed culture [Table-5 & Table-6].

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 microbial 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.[19] 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 microbial 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 Mixed Culture (*C. albicans* + Mixed Bacteria) [Table-8]. However, it did not show any inhibitory effect against *C. albicans* in Before as well as After Treatment assays [Table-7].

The standard antibiotic Ciprofloxacin showed inhibitory effect against Mixed Culture (*C. albicans* + Mixed Bacteria) only in After-treatment assay [Table- 8]. Moreover, the standard antibiotic Fluconazole showed mild inhibitory activity against *C. albicans* in Before-treatment as well as in After-treatment assays [Table- 7]. However, Fluconazole did not display any inhibitory effect against Mixed Culture in Before-treatment assay [Table- 8].

Combining antibiofilm agents with antibiotics is emerging as a promising strategy to eradicate biofilms.[20] Hence, the combinatorial Antibiofilm assay was carried out against *C. albicans* and Mixed Culture (*C. albicans* + Mixed Bacteria) at 37^0 C as well as at Room Temperature (RT) using crystal violet staining method [Table-9 & Table-10]. The combination of Ascorbic Acid + Fluconazole showed the highest combinatorial antibiofilm effect at 37^0 C against *C. albicans* [Table-9]. Furthermore, 4 different combinations prepared from Ascorbic Acid (AA), Ciprofloxacin (CP) and Fluconazole (F)- standard antibiotics were also evaluated to determine their combinatorial effect against Mixed Culture (*C. albicans* + Mixed Bacteria). The combinations were labelled as CP + F, AA + CP, AA + F and AA + CP + F. The combination of Ciprofloxacin + Fluconazole exhibited the highest combinatorial effect against Mixed Culture at RT. In general, the 4 combinations displayed higher inhibitory activity at RT against Mixed Culture [Table-10].

In general, combination AA + F revealed synergistic effect against Biofilms of *C. albicans* and Mixed Culture (*C. albicans* + Mixed Bacteria) especially at 37^0 C, whereas, combination AA + CP + F showed synergistic effect against Biofilm of Mixed Culture at 37^0 C as well as at RT.

The synergistic effect of Ascorbic acid with antibiotics could be due to its effect on certain metabolic activities associated with protein synthesis inside microbial cells making the microbial 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.[21]

Besides, the Time course study evaluates antimicrobial activity by exposing the test microorganism to antimicrobial agent over a specific time duration. It provides valuable information about the time-dependent effect of antimicrobial agent and offers information on the temporal dynamics of antimicrobial activity. To assess time kill kinetics, series of tubes containing growth medium are usually prepared with each tube containing specific concentration of antimicrobial agent. The test microorganism is then inoculated into each tube and the tubes are then incubated under controlled conditions for predetermined time intervals. At each time interval, samples are withdrawn from each tube and plated onto appropriate growth media. After incubation, presence or absence of growth is noted. Thus, impact of antimicrobial agent on the growth and viability of the microorganism over the specified time period can be assessed through this assay. Unlike static assays, the time kill kinetics assay allows for the continuous monitoring of microbial growth providing information on the rate and extent of microbial killing or growth inhibition.[11]

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, *C. albicans* and its Mixed culture (*C. albicans* + Mixed Bacteria) 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 (100 mg/ml) revealed inhibition of *C. albicans* and Mixed Culture around 48 hours and 6 hours respectively [Table-11 & Table-12].

The standard antibiotic Ciprofloxacin which was included in the present study did not inhibit Mixed Culture even at 48 hr [Table-12]. This observation correlates with MIC result of Ciprofloxacin. Likewise, the standard antibiotic Fluconazole did not inhibit Mixed Culture even at 48 hrs [Table-12]. However, it inhibited *C. albicans* within 5 min [Table-11]. This observation also correlates with MIC result of Fluconazole.

Additionally, earlier study revealed antioxidant potential of Ascorbic Acid which was detected using DPPH assay.[22] The presence of both antioxidant and antimicrobial properties in a single molecule makes them more effective.[23]

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 microbial 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.[24]

In general, Ascorbic Acid has revealed Antifungal 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 microbes to survive.[25,26] Furthermore, natural products have been used since historical times to control spread of fungal diseases, infections and food contamination. Mode of action of antifungal natural products include, cell wall damage, disrupting cell membrane, inhibiting sporulation, inhibition of ergosterol biosynthesis etc. to name a few.[27]

Besides, our previous study has revealed broad spectrum Antibacterial and Antibiofilm effects of Ascorbic acid inhibiting Gram Positive and Gram Negative Standard Strains as well as Gram Negative Clinical Isolates.[8,28]

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*.[29] which could be an added advantage to use Ascorbic acid as Antimicrobial and Antibiofilm agent.

In general, following observations were noted in the present *in vitro* study,

1) Agar well diffusion assay and Antibiofilm assay can be carried out at 37^0 C as well as at RT (20^0 C) against Mixed Culture (*C. albicans* + Mixed Bacteria).
2) The pattern of biofilm formation of Mixed Culture (*C. albicans* + Mixed Bacteria) was found to be different when the microplates were incubated at 37^0 C and at RT (20^0 C)

respectively. The biofilm was formed on the wall/sides of the microplates (wells) that were incubated at 37^0 C, whereas, Biofilm was formed as a sharp ring in the microplates that were incubated at RT (20^0 C).

3) Antibiofilm assay against *C. albicans* can be conducted at 37^0 C as well as at RT (20^0 C).

Overall, the present *in vitro* study provides foundation for utilizing Ascorbic Acid as putative Antifungal and Antibiofilm candidate against *C. albicans* and its Mixed Culture with bacteria.

CONCLUSION

In the present *in vitro* study, Antifungal and Antibiofilm potential of Ascorbic acid was tested against 2 Fungi and Mixed Culture (*C. albicans* + Mixed Bacteria) using various assays such as MIC, MBC, Agar Well Diffusion Method, Time Course study and Antibiofilm assay. The current *in vitro* study has exhibited Antifungal and Antibiofilm potential of Ascorbic acid against Mono as well as Polymicrobial Culture. Moreover, combination of Ascorbic Acid + Fluconazole revealed synergistic effect against Biofilms of *C. albicans* and Mixed Culture (*C. albicans* + Mixed Bacteria) especially at 37^0 C, whereas, combination of Ascorbic Acid + Ciprofloxacin + Fluconazole showed synergistic effect against Biofilm of Mixed Culture at 37^0 C as well as at RT. Thus, Ascorbic Acid epitomizes a promising source of effective, cheap and safe Antimicrobial and Antibiofilm agent.

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