

Original Research Article

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Inhibitory Effect of Curcumin on Biofilm Produced by Bacterial Pathogens

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ABSTRACT

Biofilm production in bacterial pathogens is a serious threat to medical management of infections. Owing to decreased penetration through biofilm matrix, most antibiotics fail to effectively eliminate these bacterial strains which continue to persist and result in emergence of drug resistance in healthcare setups. Therefore, a bioactive substance with biofilm inhibitory action is more likely to enhance the antimicrobial effect of antibiotic therapy. To investigate the anti-biofilm action of curcumin in bacterial pathogens. Biofilm producing bacterial strains isolated from various samples were identified by Congo red agar method. These isolates were inoculated on Congo red agar with increasing concentrations of curcumin (2, 4, 8, 16, 32, 64, 128, 256 and 512 µg/ml) to detect the inhibition of biofilm formation at its various concentration. Biofilm production was detected among 60 isolates i.e. *Escherichia coli* (n=23, 38.3%), *Klebsiella pneumoniae* (n=13, 21.6%), *Staphylococcus aureus* (n=13, 21.6%), *Citrobacter freundii* (n=6, 10%), *Pseudomonas aeruginosa* (n=3, 5%) and *Enterococcus faecalis* (n=2, 3.3%) by Congo red agar. Majority (53.3%) were from urine samples, followed by pus, sputum, aspirate and endotracheal secretion. These isolates showed minimum biofilm inhibitory concentration (MBIC) ranging from 4 to 128 µg/ml. Among gram negative bacteria, higher MBIC was found among *E. coli* (79.3% isolates with MBIC 128 µg/ml) and among gram positive bacteria 84.6% *S. aureus* isolates had MBIC of 32 µg/ml. Curcumin was found to be a potent bioactive substance which inhibits biofilm formation in gram positive and gram negative bacterial clinical isolates. It may have promising role in treatment of infections with biofilm forming bacteria in combination therapy with antibiotics.

Keywords

Biofilm,
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Introduction

Biofilm are complex microbial ecosystem which are adherent to a substratum or interface or to each other in a self-produced extracellular polymeric matrix. Biofilm producers represent 85% of human related infections of which 65% are health care associated (Percival *et al.*, 2015). Biofilm producing microorganisms are common

among critically ill patients increasing the mortality. They also have reduced susceptibility to antimicrobial agents. The proposed mechanisms for such reduced susceptibility includes slower growth rate of these organisms which reduces the uptake of the antibiotics and delayed penetration of the same through the matrix (Donlan, 2002).

Hence, antimicrobial resistance among the biofilm producing clinical bacterial strains make the treatment cumbersome (Reiter *et al.*, 2011) The curcumoids are a group of phytochemicals produced by *Curcuma longa* which is a rhizome with common name turmeric (Ammon, 1991). These plants are found frequently in Indian subcontinent and South East Asia (Negi *et al.*, 2014) among the curcumoids produced by these plants curcumin or diferuloylmethane has antibacterial, antiviral, antifungal and antibiofilm effect. The curcumin content of these plants varies from plant species and their geographical location (Hayakawa *et al.*, 2011). The safety of curcumin in high doses has provoked its utility in combination with various antimicrobial preparations. The aim of our study was to determine the anti-biofilm action of curcumin.

Materials and Methods

A prospective study was conducted over a period of six months from May to October 2015 in department of Microbiology of our tertiary care teaching hospital in southern part of India. Our study had two arms one was the synergistic effect of curcumin with antibiotics against biofilm, was the subject of early publication (Kali *et al.*, 2016). We present another arm of our study on the inhibitory effect of curcumin on the biofilm produced by microbes.

Bacterial strains isolated from various clinical samples were included in the study. Samples were collected with aseptic precautions and were processed as per standard laboratory procedures. The bacterial strains isolated from various clinical samples were subjected to biofilm detection and determination of minimum biofilm inhibitory concentration (MBIC) for curcumin.

Detection of biofilm production was carried out by Congo red agar method (Freeman *et*

al., 1989). The bacterial isolates were inoculated on this medium. After incubation at 37°C for 48 hours the plates were observed for biofilm production. It was indicated by presence of dry black crystalline colonies. The strains which were non biofilm producers remained pink and had no crystalline texture.

We have used Congo red agar media with increasing concentration of curcumin (agar dilution) to determine the MBIC of curcumin among various bacterial strains. Under proper aseptic precautions curcumin powder was dissolved in dimethyl sulfoxide (DMSO) and a stock solution (128 mg/L) of curcumin was prepared. Required volumes of the stock solution of curcumin were added to molten brain heart infusion (BHI) agar (with 5% sucrose and 0.08% Congo red) at 55°C to achieve final curcumin test concentration of 2, 4, 8, 16, 32, 64, 128, 256 and 512 µg/ml. All biofilm producer isolates were inoculated on Congo red agar plates with increasing concentration of curcumin. The inhibition of biofilm production was noted when an isolate which produced black crystalline colonies on Congo red agar failed to develop black crystalline consistency in the presence of curcumin.

Microsoft excel datasheet was used for tabulation and analysis of the data by calculation of percentage and the simple ratios.

Results and Discussion

A total of 400 bacterial pathogens from various samples were screened for biofilm production by Congo red agar method and 60 biofilm producing bacterial strains were identified. This includes 15 gram positive (*S. aureus* and *E. faecalis*) and 45 gram negative bacteria (*E. coli*, *K. pneumoniae*, *C. freundii* and *P. aeruginosa*). The bacterial

strains recovered from various sample and their percentage is shown in Table 1. The biofilm producer bacteria were mainly urinary (n=32, 53.3%) and pus isolates (n=22, 36.6%). Sputum, aspirate and endotracheal secretion recovered only 3, 2 and 1 biofilm producing strain respectively.

The minimum biofilm inhibitory concentrations (MBIC) of curcumin were determined in these isolates and are shown in Table 2. All isolates showed inhibition of biofilm formation at 128 µg/ml concentration of curcumin.

Medical implications of biofilm are increasingly recognized with the development of diagnostic techniques in recent years. This complex microbial organization is found to be the preferred mode of growth on living as well as non-living surfaces, rather than simple accumulation of microbial cells. Biofilm producing organisms are associated with native tissue / organ infections or indwelling device infections (Hall-Stoodley *et al.*, 2004). Central and peripheral venous catheters, urinary catheters, prosthetic heart valves and intrauterine devices are the common indwelling devices from which biofilm producers can be isolated. The native host tissue infections include periodontitis, native valve endocarditis, cystic fibrosis, chronic rhinosinusitis, otitis media, chronic prostatitis, urinary tract infections and wound infections. Several studies have evaluated the prevalence of biofilm-associated infections in various clinical setups. Around 60% of biofilm producing organism have been reported from catheter associated urinary tract infections and chronic wound infections (Niveditha *et al.*, 2012, James *et al.*, 2008) In our study, 15% (60 out of 400) of bacterial isolates were found to produce biofilm. Majority of the biofilm producing isolates

were isolated from urine samples (n=32, 53.3%), followed by pus (n=22, 36.6%), sputum (n=3, 5%), aspirate (n=2, 3.3%) and endotracheal secretion (n=1, 1.6%). We found the biofilm associated urinary infections were mostly among patients with indwelling urinary catheter (n=17). Although biofilm production is not uncommon in commensal species found in healthy hosts, formation of biofilm by pathogenic bacteria has more medical significance. Biofilm-associated infections caused by the gram-positive bacterial pathogens, such as *S. aureus* and *Enterococcus sp* and gram-negative bacilli, such as *E. coli*, *K. pneumoniae* and *P. aeruginosa* have been well documented. In a study conducted in USA, 91% *S. aureus*, 83% *P. aeruginosa*, 76% *K. pneumoniae*, 55% *Acinetobacter baumannii* and 13% of *E. coli* isolates were biofilm producers (Sanchez *et al.*, 2013). However, there were no significant differences in biofilm formation between the MRSA and MSSA strains. Similar results have been reported in other studies (Gjodsbol *et al.*, 2006, Bjarnsholt 2013) In our study, out of 60 biofilm producing isolates, majority were *E. coli* (n=23), followed by *K. pneumoniae* (n=13), *S. aureus* (n=13), *C. freundii* (n=6), *P. aeruginosa* (n=3) and *E. faecalis* (n=2). 19 of 23 *E. coli* isolates were associated with urinary infections.

From medical point of view, biofilm associated infections are persistent in nature and are less amenable to antimicrobial therapy. Biofilm producing bacteria display several fold increase in resistance against multiple antibiotics. The biofilm exopolysaccharide matrix imparts protection from antimicrobial molecules as well as from host immune defense. However, the mechanisms of antimicrobial resistance in biofilm is quite unique in comparison to its planktonic counterpart. In contrast to the

intrinsic mechanisms found in planktonic cells such as altered targets, decreased antibiotic influx, enhanced efflux and enzymatic degradation, biofilms negate the antibiotic effect by decelerating the antibiotic diffusion through its matrix as the antibiotic molecules bind to the oppositely charged biofilm polymers. In addition, supporting the growth of a subpopulations of persister cells, repression of growth in response to starvation-induced stress and glucan mediated antibiotic sequestration have also been implicated in biofilm-specific antimicrobial resistance (Stewart, 2015). Furthermore, the minimum biofilm inhibitory concentration (MBIC) may exceed the toxic concentration and limit the therapeutic use of antibiotic. Consequently, therapeutic strategies to negate biofilm came up as a new area of interest in infection control (Abedon, 2015). Among the several newly proposed therapeutics, bioactive natural products have shown promising results. Curcumin is the major active phytochemical present in the rhizome of *Curcuma longa*, commonly known as turmeric. It is well known for its medicinal values in traditional Indian and Chinese medicine. The anti-biofilm effect of curcumin has been studied both *in vivo* and

in vitro against various bacteria. Curcumin has displayed MIC values of 16 µg/mL and 128 µg/mL against *Bacillus subtilis* and *S. aureus* respectively (Ungphaiboon *et al.*, 2005). It was also found to destabilize biofilm formation by *Helicobacter pylori* at a concentration of 10µg/ml (Hegarty *et al.*, 1999). In our study, we evaluated the MBIC of curcumin against various clinical isolates *in vitro*. While 18 isolates (30%) had MBIC of 128µg/ml, a total of 8, 19, 12, 2 and 1 isolate displayed MBIC of 64µg/ml, 32 µg/ml, 16 µg/ml, 8 µg/ml and 4 µg/ml respectively. Among *E. coli*, 79.3% of isolates had MBIC of 128µg/ml and among *S.aureus* 84.6% of isolates had MBIC of 32µg/ml. We found all three *P. aeruginosa* strains isolated from pus, sputum and urine samples had MBIC of 16µg/ml which was similar to another study. They reported 16µg/ml MBIC in *P. aeruginosa* isolates from cystic fibrosis (Karaman *et al.*, 2013). Common anti-pseudomonas antibiotics such as imipenem and aminoglycosides often found to stimulate alginate production leading to increased biofilm volume in *P. aeruginosa* at their sub-MIC concentrations which may limit their therapeutic benefits.

Table.1 Sample-wise distribution of biofilm producing bacterial isolates

Organism	Urine	Pus	Sputum	Aspirate	Endotracheal secretion
<i>E. coli</i> (n=23, 38.3%)	19	4	-	-	-
<i>K.pneumoniae</i> (n=13, 21.6%)	7	4	1	-	1
<i>S. aureus</i> (n=13, 21.6%)	-	11	-	2	-
<i>C. freundii</i> (n=6, 10%)	4	1	1	-	-
<i>P. aeruginosa</i> (n=3, 5%)	1	1	1	-	-
<i>E. faecalis</i> (n=2, 3.3%)	1	1	-	-	-

Table.2 Minimum biofilm inhibitory concentration of curcumin in biofilm producing bacteria

	4 µg/ml	8 µg/ml	16 µg/ml	32 µg/ml	64 µg/ml	128 µg/ml
<i>E. coli</i> (n=23)	1	1	1	2	1	17
<i>K. pneumoniae</i> (n=13)	-	1	4	5	3	-
<i>S. aureus</i> n=13)	-	-	1	11	1	-
<i>C. freundii</i> (n=6)	-	-	3	1	2	-
<i>P. aeruginosa</i> (n=3)	-	-	3	-	-	-
<i>E. faecalis</i> (n=2)	-	-	-	-	1	1
Total (n=60)	1(1.6%)	2 (3.3%)	12 (20%)	19 (31.6%)	8(13.3%)	18 (30%)

In contrast, exposure to the sub-MIC concentrations curcumin was not associated with increased biofilm production (Karaman *et al.*, 2013).

The limitation of our study is less number of samples tested. We have used Congo red agar method for biofilm detection, whereas other methods like tube adherence test, micro titer plate method, scanning-electron microscopy were not used for confirmation. The low prevalence of biofilm producing bacteria in our study may be attributed to the less sample size, difference in method of biofilm detection, sample types and sites of infection, regional variation of strain types and infection control practices.

In conclusion, curcumin is a potent bioactive substance. In addition to its antimicrobial action, it has anti-biofilm effect. In our study, it was found to inhibit biofilm formation in gram positive as well as gram negative clinical bacterial isolates. Biofilm formation of all pathogenic bacterial isolates were inhibited at 128 µg/ml concentration of curcumin. Hence, it may have promising role in treatment of infections with biofilm forming microorganism as an adjunct to antibiotic therapy, especially in case of multidrug resistant organisms where the therapeutic options are limited to only a few antibiotics.

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