

Asian Journal of  
**Biotechnology**

## Study on Effect of Different Plant Extracts on Microbial Biofilms

<sup>1</sup>B. Sasirekha, <sup>2</sup>D.M. Megha, <sup>2</sup>M.S. Sharath Chandra and <sup>2</sup>R. Soujanya

<sup>1</sup>Department of Microbiology, Acharya Bangalore B School, Andrahalli Main Road, Magadi Road, Bangalore, Karnataka, India

<sup>2</sup>Department of Microbiology, Jain University, Jayanagar, 3rd Block, Bangalore, Karnataka, India

*Corresponding Author: B. Sasirekha, Department of Microbiology, Acharya Bangalore B School, Andrahalli Main Road, Magadi Road, Bangalore, Karnataka, India*

### ABSTRACT

With the emergence of strains resistant to conventional antibiotics, it is important to carry studies using alternative methods to control these microorganisms causing important infections, such as the use of products of plant origin that has demonstrated effective antimicrobial activity besides biocompatibility. Therefore, in the present study an attempt has been made to screen different plant extracts against microbial biofilms. Out of 33 isolates BF5 was found to be a high biofilm producer by crystal violet assay with high hydrophobicity. On studying the factors influencing biofilm production increase in glucose concentration, acidic and alkaline pH, high osmolarity and low iron concentration promoted BF 5 biofilm formation. Among the different plant extracts tested, turmeric showed 100% efficacy against BF5 followed by cloves (97%), Indian borage (91%) and *Aloe vera* (84%). Turmeric and cloves extracts exhibited 50% antibiofilm activity against preformed biofilm.

**Key words:** Biofilm, turmeric, hydrophobicity, crystal violet assay

### INTRODUCTION

In the past few decades, use of medicinal plants for their primary health care has gained importance in developing countries (Namasivayam and Avimanyu, 2011). Herbal extracts or essential oils prepared from medicinal plants contain different compounds with numerous biological activities confirmed by *in vitro* and *in vivo* studies, such as antibacterial, antifungal, antiviral, antiprotozoal, anthelmintic, antiseptic, anti-inflammatory, antitumor, antioxidative, antiallergic, anticonvulsant, antidepressant, contraceptive, antimutagenic, analgesic and diuretic properties (Haidari *et al.*, 2009). Scientific knowledge of the antimicrobial properties of plant species may contribute to the development of drugs against pathogenic microorganisms.

Biofilms, or surface-attached communities of cells encapsulated in an extracellular matrix, represent a common lifestyle for many bacteria. Bacterial cells within a biofilm often exhibit altered physiology, including enhanced resistance to antibiotics and other environmental stresses (Branda *et al.*, 2005). Biofilm promotes bacterial persistence by resisting, antibiotic treatment and host immune responses (Namasivayam *et al.*, 2012). Biofilm formation renders antibiotics ineffective due to their relative impermeability, variable physiological status of microorganisms, subpopulations of persistent strains and variations of phenotypes present (Hall-Stoodley *et al.*, 2004). Biofilms have been reported to show increased resistance to antimicrobial agents viz., antibiotics compared to free-floating cells.

Alternative microbiological control measures that are suitable for use in humans are of the utmost importance in view of the emergence of antimicrobial-resistant strains. Therefore, the present study evaluated the effectiveness of herbal extracts of *Aloe vera* (Aloevera), *Curcuma longa* (turmeric), *Plectranthus amboinicus* (Indian borage) and *Syzygium aromaticum* (cloves) leaf extracts against a biofilm forming strain was analyzed.

## MATERIALS AND METHODS

Ten soil samples were collected from different locations of in and around Bangalore and packed in a sterile polythene bag and labeled properly. The soils samples were stored at 4°C until further processed.

**Screening for biofilm producers:** One gram of soil sample was weighed and serially diluted up to  $10^{-9}$  dilution in sterile distilled water. One milliliter of the respective dilutions were plated onto congo red agar plates (37 g brain heart infusion, 20 g agar, 50 g sucrose and 0.8 g congo red per litre of distilled water) and incubated at 37°C for 24-48 h. Crusty black colonies with dry filamentous appearance were recorded as biofilm producers and smooth pink colonies as non-producers (Arciola *et al.*, 2005).

**Determination of biofilm production:** Semi-quantitative assessment of biofilm formation was assessed using the modified Crystal Violet (CV) assay (Djordjevic *et al.*, 2002). The CV assay is based on the principle that the dye penetrates and binds to negatively charged extracellular molecules, including cell surface molecules and polysaccharides in the extracellular matrices in mature biofilms thereby providing information on the density of the attached cells. Briefly, 180 µL of tryptone soy broth (TSB, Himedia) and 20 µL of bacterial suspension ( $10^7$  CFU mL<sup>-1</sup>) of BF5 isolate was added to microtitre plate of 96 wells and TSB broth alone was used as negative control. Microtitre plate was incubated at 37°C for 24 h. After incubation media was removed from the microtitre plate by inversion and wells were gently washed with sterile distilled water and cells adhered to the microtitre plate were stained with 200 µL of crystal violet solution (0.1%) for 30 min. The dye was discarded and microtitre plates was dried at 40°C for 15 min. Biofilm was quantified by adding 200 µL of 95% of ethanol to each well and the OD was measured at 595 nm using Elisa reader (Lisa plus, Germany) after the adjustment to zero of the negative control. Strains were considered as efficient in biofilm formation when absorbance at 595 nm was equal or the greater than 0.15 (Di Martino *et al.*, 2003). The experiment was performed in triplicate and the mean OD value was considered.

**Bacterial hydrophobicity:** Bacterial hydrophobicity was assayed by the ammonium sulphate method. Bacterial suspension (15 µL) was combined with different concentrations of ammonium sulphate (0.5, 1, 1.5, 2, 2.5, 3, 3.5 and 4.0 M) (15 µL) on a glass slide. The suspension was gently mixed and observed for aggregate formation for 2 min. Agglutination in salt concentrations of  $\leq 1.5$  M indicated surface hydrophobicity (Sherman *et al.*, 1985).

**Biofilm quantification:** The LPDE (Low-density polyethylene) films of 3×3 cm dimension were taken and disinfected with 70% ethanol for 30 min, washed with distilled water for 10 min. Disinfected LPDE films was added into 100 mL of LB (Luria Bertani) media inoculated with 24 h

culture. Media was incubated at 37°C. After every 10 days, LPDE film was taken washed in 10% ethanol by vigorous shaking. The 0.1 mL aliquot was spread plated onto LB plates. After incubating the plates at 28±1°C for 24 h, CFU mL<sup>-1</sup> was calculated (O'Toole *et al.*, 2000).

**Biochemical identification of bacterial strain:** Isolate BF 5 exhibiting maximum biofilm potential was subjected to cultural, morphological and biochemical test as described in Bergey's manual of systematic Bacteriology (Garrrity *et al.*, 2004).

**Antibiotic susceptibility profile:** The antimicrobial susceptibility patterns of isolate to different antimicrobial agents were determined by using disc diffusion test and interpreted according to CLSI (CLSI., 2012). Antibiotics tested were ciprofloxacin, amikacin, tetracycline, polymixin-B, gentamicin, imipenam, cefepime, rifampicin, chloramphenicol, ceftazidime, erythromycin, cefuroxime and cefoxitin.

**Factors influencing the biofilm formation:** Biofilm formation of bacteria under various culture conditions was determined as described by Taweekaisupapong *et al.* (2005). Different wells of 96 well microtiter plate were inoculated with 100 µL of BF5 isolate culture (10<sup>8</sup> CFU mL<sup>-1</sup>) and 100 µL of M9 media supplemented with different concentrations of glucose (2, 10, 50, 100, 150 and 200 mM); pH (5.0, 6.9, 7.2, 8.0 and 9.0); temperature at 4, 25 and 37°C; NaCl (0.085, 1.19, 1.36, 1.53 and 1.7 M) and FeSO<sub>4</sub>.7H<sub>2</sub>O (10, 25, 50, 100, 500 and 1000 mM). After incubation media was removed from the microtitre plate by inversion and wells were gently washed with sterile distilled water and cells adhered to the microtitre were stained with 200 µL of crystal violet solution (0.1%) for 30 min. The dye was discarded and microtitre plates was dried at 40°C for 15 min. Biofilm was quantified by adding 200 µL of 95% of ethanol to each well and the OD was measured at 595 nm using Elisa reader (Lisa plus, Germany) after the adjustment to zero of the negative control. Strains were considered as efficient in biofilm formation when absorbance at 595 nm was equal or the greater than 0.15 (Di Martino *et al.*, 2003). Experiment was repeated in triplicate.

**Antimicrobial screening of plant extracts:** Leaves of plants *Aloe vera*, Indian Borage, turmeric and cloves were obtained and air-dried at room temperature and then ground into fine powders which were stored into airtight containers at room temperature. Powdered plant material (1 g) was weighed into a conical flask and 10 mL of distilled water added to cover the powder. The flask was sealed with foil and incubated at 45°C in a water bath for 3 h with intermittent shaking. Following incubation, the extract was filtered using funnel through Whatman (No. 1) filter paper. The extraction process above was repeated twice on the residue using 5 mL of water at the second extraction. The filtrates obtained from the filtration process were pooled, dried and stored at 4°C.

**Minimum inhibitory concentration:** To examine the inhibitory effect of test agents (*Aloe vera*, Indian Borage, turmeric and cloves) on the biofilm growth, modified crystal violet assay was carried out. Water extracts were dissolved in sterile distilled water to a stock concentration of 200 mg mL<sup>-1</sup>. The positive control for bacterial cultures was ciprofloxacin. Controls were prepared at a stock concentration of 0.01 mg mL<sup>-1</sup>. Briefly, 100 µL of sterile distilled water was aliquoted into all the wells of the microtitre plate. The prepared extracts were then pipetted into the wells A1-A12 of the plate. Doubling dilutions were performed in the direction A to H resulting in decreasing concentrations, following doubling dilutions, 100 µL of the BF 5 bacterial culture (0.5 McFarland)

was added to all the wells. The plates were incubated at 37°C for 24 h for all bacterial strains. Water was used as negative control. Following incubation, the crystal violet assay was performed to assess biomass of the attached cells. The percentage of biofilm inhibition was calculated using the following equation (Wei *et al.*, 2006):

$$1 - \frac{\text{OD}_{590} \text{ of cells treated with test agent}}{\text{OD}_{590} \text{ of non-treated control}} \times 100$$

**Inhibition of a preformed biofilm:** Inhibition of a preformed biofilm was investigated using plant extracts that exhibited >50% inhibition of cell attachment. Biofilm formation was achieved by aliquoting 100 µL of (0.5 McFarland) culture into a 96 well microtitre plate. The plates were then incubated at 37°C for 4 h to allow cell attachment. Following the 4 h incubation, 100 µL of each plant extract was added to a final concentration of 200 mg mL<sup>-1</sup> in the wells and ciprofloxacin was used as the positive control. The plates were further incubated at 37°C for 24 h. Following incubation with the extracts, the crystal violet assay was performed as described above.

## RESULTS AND DISCUSSION

Biofilm and multidrug resistance have been identified as virulence factors of great magnitude in clinical infections. Due to the increase in complexity of most microbial infections and the resistance to conventional therapy, researchers have been prompted to identify alternatives for the treatment of infections. Plant extracts and other biologically active compounds isolated from plants have gained widespread interest in this regard as they have been known to cure diseases and illness since ancient times.

From 5 different soil samples, a total of 33 isolates were obtained out of which 3 isolates showed black dry crystalline colonies after 24 h of incubation and 6 isolates produced black coloured colonies after 48 h of incubation whereas 24 isolates were non biofilm producers developed red colonies on congo red agar plates.

Production of biofilm in bacterial isolates from both clinical and environmental samples has been described by Smith *et al.* (2008). Abraham *et al.* (2012) in his study isolated a high biofilm yielding strain from soil sample. Gutierrez *et al.* (2012) demonstrated biofilm forming ability of 63 *S. aureus* isolates isolated from 442 environmental samples on congo red plates and by PCR amplification of genes involved in biofilm formation. Li *et al.* (2003) in his study showed O33 stain to be high biofilm producer by both tetrazolium reduction assay and by crystal violet assay and stated crystal violet staining method to be accurate and faster. Saxena *et al.* (2014) reported modified tissue culture plate method to be accurate screening method as compared to other methods for studying biofilm formations.

**Semi-quantitative assessment of biofilm formation:** The CV staining method is widely used for measuring biofilms in bacteria (Djordjevic *et al.*, 2002). In our study quantitative analysis of biofilm formation in 9 isolates by crystal violet assay demonstrated BF 5 to be a high biofilm producer with an OD value of 0.022. Further crystal assay also revealed BF 5 isolate possess capacity to form biofilm on plastic surfaces. Our results are in agreement with that of Agarwal *et al.* (2011) and Hassan *et al.* (2011) who suggested microtitre plate assay to be effectively used for the assessment of biofilm ability.

**Hydrophobicity:** Hydrophobic materials are reported as surfaces that provide a greater bacterial adherence (Djordjevic *et al.*, 2002). Strain possessing cell surface hydrophobicity has been attributed the most important in biofilm formation. The BF 5 isolate demonstrated cell surface hydrophobicity by aggregating ammonium sulfate up to 3 M.

Hydrophobic interaction has been thought to be a major event in the attachment of bacteria to host cells, indwelling medical prosthetic devices and to plastics used in nutritional devices. Many prior studies suggested that hydrophobic interactions contribute to the initial binding of pathogens to tissues, leading to colonization, invasion or tissue destruction (Kouidhi *et al.*, 2010). Microorganisms adhere to a substratum via the hydrophobic effect if the associating sites possess sufficiently high densities of apolar areas (Doyle, 2000). Agarwal *et al.* (2011) in his study used plastic surface for biofilm formation and found it be efficient. Thus BF5 isolate may be more prone to binding to hydrophobic surfaces.

**Quantification of biofilm:** Biofilm forming ability of the BF 5 isolate increased with the duration of incubation period. The BF 5 isolates colonizing LDPE films increased dramatically up to 30 days (Fig. 1). The high hydrophobic nature of BF 5 isolate might have helped for colonization. As adhesion is the first step in complex process of biofilm formation this could be one of the possible explanations for the ability of the bacteria to produce biofilm in high number in plastic surfaces. The duration of incubation period also considerably influenced the amount of biofilm. The results of our study are in agreement with other studies (Malcova *et al.*, 2008). Increase on biofilm concentration upto 30 days indicated high hydrophobicity and also indicated that biofilm has not reached maturation. Besciak and Surmacz-Gorska (2011) stated conventional plate method to be superior to absorbance measurement.

**Factors influencing biofilm formation:** Several environmental factors, such as glucose, osmolarity, ethanol, temperature and anaerobiosis have been reported to affect biofilm formation.

**Effect of glucose:** Biofilm forming capacity of BF 5 when evaluated in the presence of different concentration of glucose showed increase in biofilm production with increasing concentrations of glucose in the medium. Glucose supplementation has been shown to influence biofilm formation (Fig. 2a). Marinho *et al.* (2013) demonstrated a synergistic effect between glucose and biofilm formation in antibiotic-resistant *E. faecalis* and *E. faecium*. The association of the glucose in the

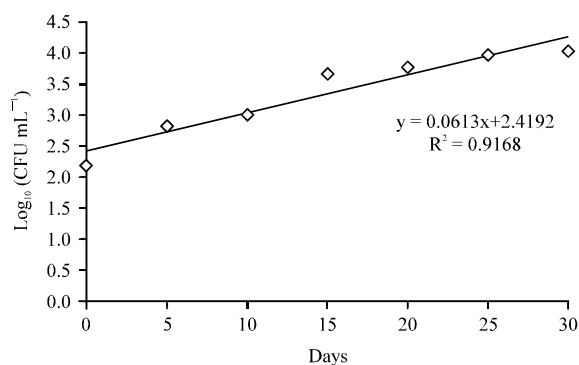


Fig. 1: Quantification of biofilm formation in BF 5

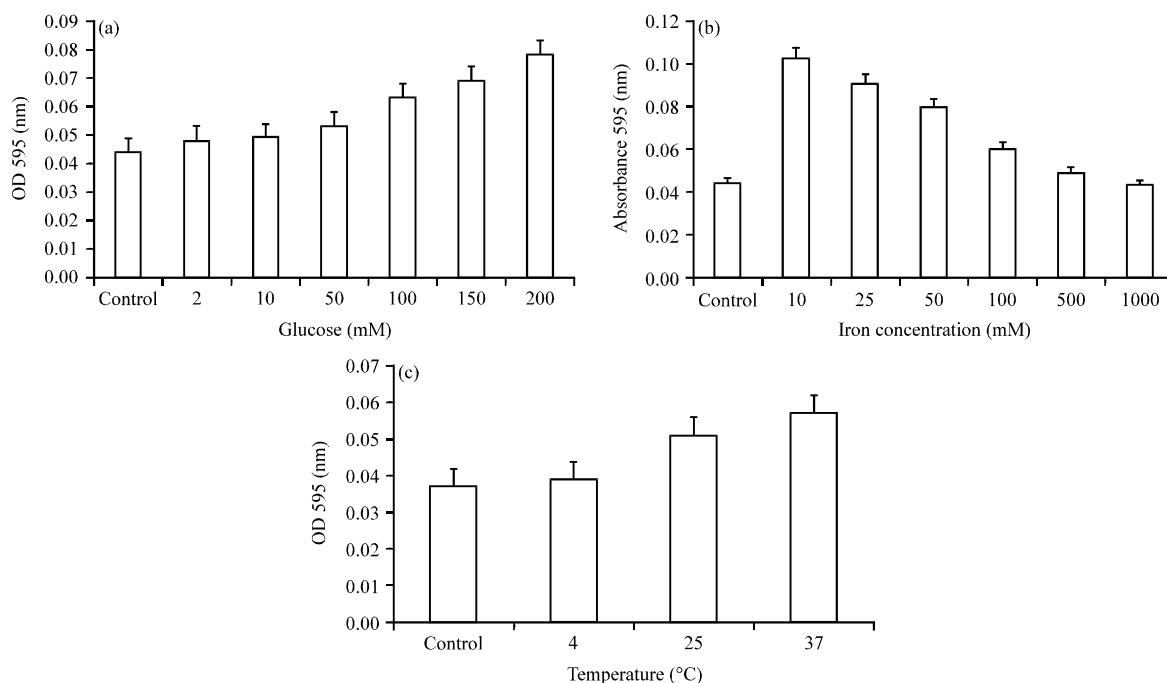


Fig. 2(a-c): Effect of (a) Glucose, (b) Iron and (c) Temperature on biofilm formation

medium and the capacity of biofilm formation have been reported in several bacterial species (Sousa *et al.*, 2008). Our results are consistent with the findings of Baldassarri *et al.* (2001).

**Effect of iron:** Supplementation of media with different concentration of iron showed a decrease in biofilm formation as iron concentration increased suggesting that biofilm formation is negatively controlled by iron (Fig. 2b). Similarly, Yang *et al.* (2007) stated that biofilm formation were favoured in media with low iron concentrations (5  $\mu\text{M}$   $\text{FeCl}_3$ ). In *S. aureus*, iron depletion was shown to promote biofilm formation and this response was partially regulated by Fur genes. (Johnson *et al.*, 2005) whereas Cai *et al.* (2010) demonstrated iron-depletion can retard biofilm formation. Singh (2004) in his study showed thicker biofilms in the absence of lactoferrin in *P. aeruginosa*.

**Effect of temperature:** The BF 5 grown at 25 and 37°C displayed increase in biofilm formation whereas incubated at 4°C showed less growth (Fig. 2c). It has been suggested that the increased hydrophobicity at high temperatures (37°C) may enhance the initial cell adherence, contributing to a higher biofilm density (Di Bonaventura *et al.*, 2008). Further it is not surprising that bacteria form more biofilm at 37°C than in other temperatures tested since at this temperature bacteria grow best and consequently the cells number increase and as a result, the cell mass facilitates the sedimentation, resulting in a higher degree of initial attachment. Lower absorbance caused at 18°C can be caused by a slower bacterial growth. Marinho *et al.* (2013) demonstrated 98.46 and 100% biofilm formation by *E. faecalis* and *E. faecium* in polystyrene plates.

**Effect of pH:** Biofilm production at slightly acidic and alkaline pH was comparably higher than at neutral pH (Fig. 3a). Ability of the isolate to withstand acidic pH is an important adaptive

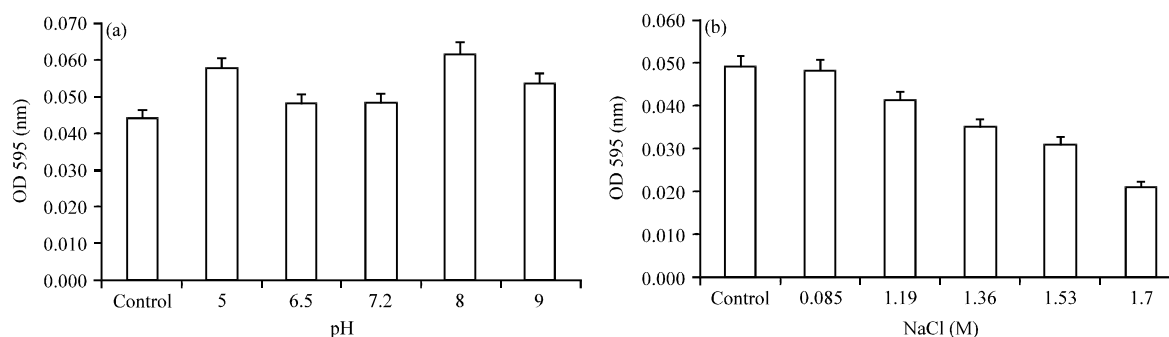


Fig. 3(a-b): Effect of (a) pH and (b) Sodium chloride on BF 5 in biofilm development

response. Changes in external pH can significantly influence many physiological parameters, such as energy coupling, ion transport, proton movement and export of metabolic products, thereby triggering numerous secondary signals.

The data obtained in our study was in accordance with Kayaoglu *et al.* (2005) where they found increase in pH above 8.5 caused decrease in adherence capacity of *E. faecalis* on bovine serum albumin and collagen type I coated wells. Similarly Manikandan *et al.* (2013) showed that *E. faecalis* has the ability to form biofilm and survive at all alkaline pH's tested (7.3-12.3).

**Effect of NaCl on BF 5 biofilm development:** In the present study as the concentration of sodium chloride in the culture medium increased, a marked decrease in biofilm production was observed (Fig. 3b). This finding is consistent with that of Havasi *et al.* (2008) who demonstrated inhibitory effect of hypertonic saline on *P. aeruginosa* motility and growth. On the other hand Kapfhammer *et al.* (2005) stated that *Vibrio cholerae* can form a biofilm under high salt conditions. Species-specific differences in the regulation of genes involved in biofilm production may account for the observation.

**Identification of bacterial isolate:** The isolate produced smooth, circular, undulate margin with flat creamish colonies on nutrient agar. Isolate was found to be Gram-positive and rod-shaped bacteria, non-motile, tested positive for catalase and negative for oxidase. Based on Bergey's Manual of Systematic Bacteriology, the isolate was identified as genus of *Bacillus* sp.

**Antibiotic susceptibility plate:** The BF 5 isolate was highly susceptible to cefuroxime, cefoxitin, imipenem ciprofloxacin, chloramphenicol, rifampicin, tetracycline and gentamicin and resistance to penicillin, amoxicillin, ampicillin ceftazidime, aztreonam. Our results are in line with these findings. Eyoh *et al.* (2014) reported no significant difference in the percentage of MDR (Multi Drug Resistance) among biofilm and non-biofilm producers.

Fitzpatrick *et al.* (2005) in his study reported more MDR stains among biofilm producers than non-biofilm formers. Ebrahimi *et al.* (2013) showed low sensitivity to streptomycin, fluoroquinolones and kanamycin by *S. agalactiae* isolates which were reported to be biofilm producers.

The MDR in biofilm has been partly attributed to the extracellular polymeric substances constituting this matrix serving as a diffusional barrier for antibiotics, thus influencing either the rate of transport of the molecule to the biofilm interior or the reaction of the antimicrobial material with the matrix material.



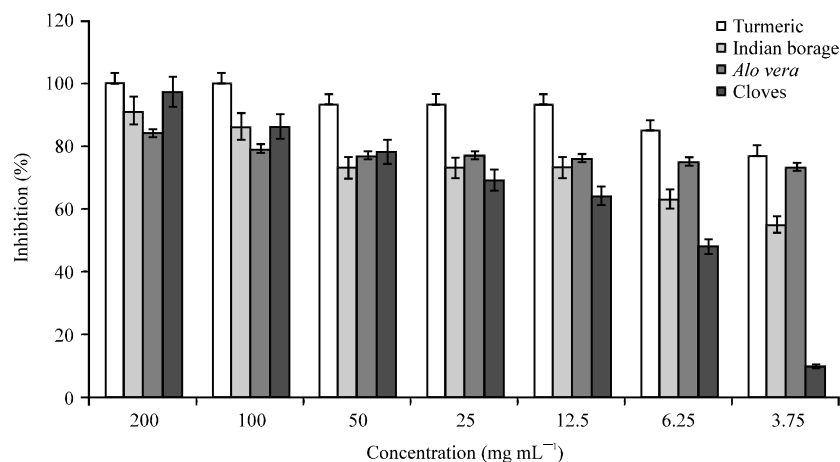


Fig. 4: A comparison of MIC values for water extracts against BF 5

**Antimicrobial screening of plant extracts:** Water extract of 4 plants showed potential *in vitro* activities against BF 5. The efficacy of water extract of these plants at the concentrations of 50 mg mL<sup>-1</sup> was much lower than that of concentration of 100 mg mL<sup>-1</sup> except turmeric. Turmeric extract (50 mg mL<sup>-1</sup>) showed 100% *in vitro* efficacy against BF 5. Comparing the average inhibition percentage of 4 extracts, turmeric and cloves were found to be more effective. Extracts turmeric (100%), cloves (97%), Indian borage (91%) and *Aloe vera* (84%) activities were comparable to ciprofloxacin (95%). These results show that turmeric was more active than the positive control (Fig. 4).

This is in conformity to works by Gur *et al.* (2006) and Chandarana *et al.* (2005) who suggested that aqueous turmeric extract is effective against *B. subtilis*, *E. coli* and *S. aureus* due to the presence of a phenolic compound, curcuminoid whereas Cowan (1999) showed better results in ethanolic extracts as compared to aqueous stating that organic dissolves more organic compounds resulting in the release of greater amount of active antimicrobial components.

Plant materials are ground in water, a number of phenolases and hydrolases are released and these enzymes might serve to modulate the activity of the active compounds in the extract which might be responsible for high inhibition percentage in our study. Herbalist usually uses water to prepare infusions and decoctions. A recent report has also suggested that curcumin in aqueous preparations exhibits phototoxic effect against both Gram positive and Gram negative bacteria (Neelakantan *et al.*, 2011).

The antimicrobial activity of aqueous extracts could be due to anionic components such as thiocyanate, nitrate, chlorides and sulphates in addition to many other compounds naturally present in plants (Darout *et al.*, 2000).

**Inhibition of growth of a preformed biofilm:** Efforts to prevent the development of biofilms are currently enjoying priority with researchers targeting the prevention of cell attachment or complete eradication of an already existing biofilm. Keeping this view extracts that showed at least 50% inhibition was used in the preformed biofilm assay. Turmeric and cloves extracts exhibited good antibiofilm activity against BF 5 with percentage inhibition greater than 50% (Fig. 5). These results show that inhibition of biofilm growth proved to be more difficult to achieve than cell attachment.

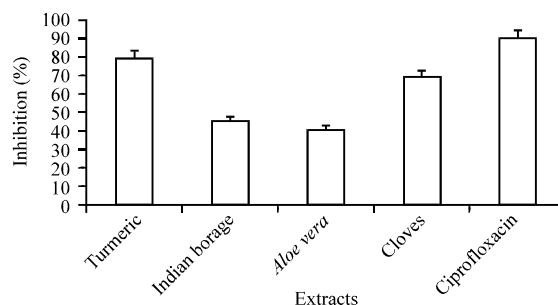


Fig. 5: Effect of plant extracts on preformed biofilms

The ease with which the plant extracts inhibited cell attachment is confirmation of previous reports where it was found that inhibition of cell attachment to a substrate is easier to achieve than inhibiting the growth of an already established biofilm (Cerca *et al.*, 2005). Turmeric and cloves successful in inhibition of preformed cell attachment can be postulated that pretreatment of the surface with plant extracts produced an unfavourable film that repel the cells back into the fluid phase, thereby reducing surface adhesion. Hashimoto (2001) in his study demonstrated the success of coating medical devices with biocides such as silver to reduce microbial adhesion.

## CONCLUSION

In conclusion we can say that, phenotypic biofilm characterization by CRA plates assay demonstrated a simple, rapid, cheap and reliable approach for detection of biofilm producers. Turmeric as was capable of microbial inhibition on a commendable scale and this may hopefully be an indication of its potential future as part of antimicrobial strategy. Investigation on large amount of clinical, environmental and commensal strains is required to understand the survival strategies biofilm producers.

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