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Chloroform extract of turmeric inhibits biofilm formation, EPS production and motility in antibiotic resistant bacteria

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Sumreen Hayat,[1,3,*] Anjum N. Sabri,[1] and Timothy D. McHugh[2]

[1] *Department of Microbiology and Molecular Genetics, University of the Punjab, Quaid-e-Azam Campus, Lahore 54590, Pakistan*
[2] *Center for Clinical Microbiology, Royal Free Campus, University College London, Rowland Hill Street, London NW3 2QG, U.K.*
[3] *Department of Microbiology, Government College University, Faisalabad, Pakistan*

In the form of biofilms, bacteria exhibit more resistance to antibiotics. Biofilm formers can withstand severe conditions and the host's defense system. Therefore, it is necessary to search for effective biofilm inhibitors. In this study, we investigated the effect of a chloroform extract of turmeric on biofilm formation against antibiotic resistant bacteria. The extract exhibited its antibiofilm effect by altering adherence, motility, extracellular polymeric substance (EPS) production and cell surface hydrophobicity; important attributes of biofilm formation. Cell attachment assays indicated that a chloroform extract resulted in a 38.9–60.2% inhibition of cell adherence to a polystyrene surface, and a 44.5–58.3% inhibition to a glass surface. Static biofilm formation assays indicated that a chloroform extract resulted in a 23–74.5% reduction in biofilm formation. The chloroform extract inhibited flagella-directed swarming and swimming motility and pilus-directed twitching motility in a dose-dependent manner. In addition to repression of motility, a chloroform extract also significantly ($p < 0.05$) altered the hydrophobic behavior, and bacterial strains such as *K. pneumoniae* and *E. cloacae* exhibited hydrophilic behavior after the addition of the extract, as compared with control cells. The presence of the extract also significantly ($p < 0.05$) increased the detachment of biofilms by a surfactant as compared with controls. Fourier transformed infrared spectroscopy (FTIR) had indicated a loss of vital functional groups of polysaccharides and proteins from the EPS of cells treated with a chloroform extract. Gas chromatography mass spectrometry (GC-MS) analysis indicated the presence of many phytochemical constituents, mainly sesquiterpenes and fatty acid groups. These results clearly suggested that turmeric could affect multiple cellular activities in biofilm formers exhibiting antibiotic resistance by modulating adherence, EPS production, motility and surface hydrophobicity.

Key Words: antibiotic resistance; biofilms; cell surface hydrophobicity; EPS; motility; turmeric

Introduction

Establishment of drug resistance among common pathogens due to poor antibiotic stewardship has made the drugs ineffective for common infections (Dimopoulus and Falagas, 2007). A key element contributing to the resistance exhibited by bacteria is the formation of biofilms. Biofilm-forming bacteria are known to exhibit increased resistance to antibacterial agents, probably due to the presence of an extracellular polymeric substance (EPS) that prevents drug penetration. EPS is mainly composed of polysaccharides, proteins, nucleic acids, metal ions and humic substances (Hall-Stoodley et al., 2004; Kives et al., 2006). Besides poor penetration, other critical factors contributing towards the reduced antibiotic susceptibility by biofilm formers include slower growth, depletion of nutrients and the establishment of persister cells (Stewart, 2002). Currently, a major concern faced by clinicians is the establishment of multidrug resistant strains having the potential to form biofilms. Therefore, there is an immediate need to develop drugs capable of treating infections caused by these pathogens.

*Corresponding author: Sumreen Hayat, Department of Microbiology, Government College University, Faisalabad, Pakistan.
E-mail: sumreenhayat@gcuf.edu.pk

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The organisms present as biofilms can be either attached or may be moving (for example, swimming and swarming). The adherence of bacteria to any surface is affected by a number of factors such as the surface (hydrophobicity), ionic strength, physiochemical nature of the environment (temperature and pH) and several attributes of micro-organisms (hydrophobicity, motility and flagellation) (Chavant et al., 2002; Folsom et al., 2006; Gorski et al., 2003; Herald and Zottola, 1988; Moltz and Martin, 2005; Ofek and Doyle, 1994). In order to control biofilms formed by drug resistant pathogens, any of these critical attributes can be targeted.

A number of bioactive compounds exhibiting antibacterial activity have been synthesized and some of them have been subjected to clinical testing to check their efficacy (Freire-Moran et al., 2011) but new compounds are still needed particularly those having the potential to inhibit biofilms. *Curcuma longa*, or turmeric, is an evergreen herbaceous plant belonging to the Zingeberaceae family that is cultivated widely in India, China, and some countries in South America (Scartezzini and Speroni, 2000). The rhizome of turmeric comprises two major classes of secondary metabolites: essential oils and phenolic curcuminoids (Funk et al., 2010). A number of important pharmacological features are attributed to these metabolites (Raina et al., 2005). Curcumin, a major and widely studied curcuminoid, gives the yellow colour to turmeric and is responsible for a number of biological activities, as are the essential oils (Hatcher et al., 2008; Menon and Sudheer, 2007). Studies have indicated that oils extracted from the leaves and floral part of turmeric plants mainly comprise monoterpenes, whereas those obtained from the rhizomes and roots mainly comprise sesquiterpenes (Ongwesa et al., 2014).

The present study aimed to determine the antibiofilm effect of the chloroform extract of turmeric against antibiotic-resistant bacterial strains in relation to cell adhesion, EPS production, cell surface hydrophobicity, and motility. In addition, the effect of chloroform extracts of turmeric on the biofilm detachment efficiency of surfactants, such as sodium dodecyl sulfate (SDS), was also elucidated. Since chloroform extraction might result in the fractionation of the majority of the hydrophobic components, including terpenoids and curcuminoids, the extract was used to determine the effect of these fractions on various attributes of biofilm formation. Biofilms are associated with the establishment of drug resistance, and, therefore, the purpose of the present study was to eliminate the formation of biofilms and their associated attributes by using a chloroform soluble fraction from turmeric.

Materials and Methods

Bacterial strains, culture media and growth conditions. Five antibiotic resistant strains were used in the present study. The strains were *Escherichia coli* (KT273995), *Enterobacter cloacae* (KT273994), *Klebsiella pneumoniae* (KT273996), *Staphylococcus aureus* (KT250728), and *Bacillus subtilis* (KT236337). The bacteria were sub-cultured in Luria-Bertani (LB) broth and on LB agar (supplemented with 1.5% agar). In order to assess the effect of the plant extract on different types of motility, swim, twitch, and swarm media were prepared as follows. Swarm medium (0.8% nutrient broth and 0.5% agar supplemented with 0.5% D-glucose), Swim media (1% tryptone, 0.5% NaCl and 0.3% agar and twitch media (1% tryptone, 0.5% yeast extract, 0.5% supplemented with 1.0% agar). Swarm plates were dried for 1 h before inoculation, whereas twitch and swim plates were dried overnight. All media, reagents and chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA) and Merck (Darmstadt, Germany).

Preparation of chloroform extract of turmeric. Turmeric rhizomes were purchased from a local market in Lahore, Pakistan. They were manually washed to remove dust and adhering sand and spread to dry. The dried rhizomes were ground by using a kitchen blender (Merlin, Austria). Extraction of 100 grams of powdered turmeric was carried out using 1000 ml chloroform, and the mixture was placed in an ultrasonic bath for 1 h and then filtered. The filtrate was concentrated by evaporating the solvent by a rotary evaporator (Heidolph, Germany) under reduced pressure at 40°C to obtain a chloroform extract of 2.66 g (2.66%). The activity of the turmeric extract was determined by using minimum inhibitory concentration (MIC) assay following the method of Smith et al. (2005). The powdered extract was completely dried to ensure the absence of any traces of the solvent and was dissolved in DMSO (dimethylsulfoxide) and then further diluted in LB broth to obtain the desired concentrations. DMSO was used at a concentration of 0.1% (v/v), ensuring no interference with each experimental system. In each experiment, control groups treated with 0.1% DMSO were also included.

GC-MS profiling of the chloroform extract of turmeric. Volatile compounds from the chloroform extract of turmeric were identified using a GC-MS analyzer (Shimadzu QP-2000). The initial temperature was maintained at 100°C for 6 min, and was increased at a rate of 10°C per min to 250°C at a bombardment energy of 70 eV. Helium was used as the carrier gas and the injection volume used was 1 μl.

Antibiotic resistant profiling. Antibiotic susceptibility testing of each strain was carried out by a disc diffusion method using Muller-Hinton agar following the recommendations of the National Committee for Clinical Laboratory Standards (Wayne, 2000). Antibiotics used were as follows: Ampicillin (10 μg), Augmentin (10 μg), Ceftriaxone (30 μg), Chloramphenicol (30 μg), Ciprofloxacin (5 μg), Ertapenum (10 μg), Erythromycin (15 μg), Oxacillin (10 μg), Penicillin (10 μg), Temocillin (30 μg), Tetracyclin (30 μg) and Vancomycin (5 μg). The discs were obtained from Oxoid (England).

Effect of the chloroform extract of turmeric on cell attachment. The effect of the chloroform extract of turmeric on the bacterial attachment to the polystyrene surface of a 96-wells microtiter plate was assessed following the method of Rodrigues et al. (2006), whereas the attachment of cells to a glass surface was determined using the protocol of Hamada and Torii (1978) with some alterations. Briefly, the chloroform extract of turmeric (100 μl) dissolved in DMSO and further diluted in LB broth was

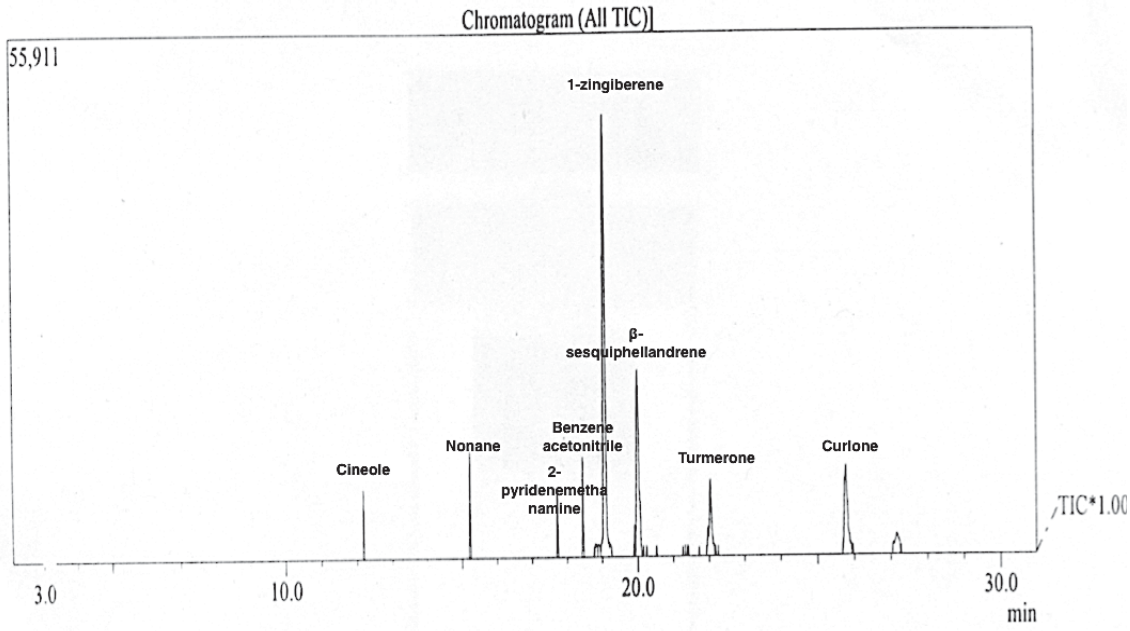

Fig. 1. GC-MS chromatogram showing phytochemicals identified in the chloroform extract of turmeric.

Table 1. Table showing antibiotic resistant profiling of bacteria used in the study.

Antibiotics used	Strains used in antibiotics susceptibility testing				
	B. subtilis	*S. aureus*	*K. pneumoniae*	*E. coli*	*E. cloacae*
Ampicillin	Resistant	Resistant	Resistant	Resistant	Resistant
Augmentin	Susceptible	Resistant	Resistant	Resistant	Resistant
Ceftriaxone	Susceptible	Resistant	Resistant	Resistant	Resistant
Chloramphenicol	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
Ciprofloxacin	Resistant	Resistant	Resistant	Resistant	Resistant
Ertapenum	Susceptible	Resistant	Susceptible	Susceptible	Susceptible
Erythromycin	Resistant	Susceptible	Resistant	Resistant	Resistant
Oxacillin	Resistant	Resistant	Resistant	Resistant	Resistant
Penicillin	Resistant	Resistant	Resistant	Resistant	Resistant
Temocillin	Resistant	Resistant	Susceptible	Resistant	Resistant
Tetracyclin	Susceptible	Susceptible	Susceptible	Resistant	Resistant
Vancomycin	Susceptible	Resistant	Resistant	Resistant	Resistant

added to the wells at a sub-inhibitory concentration (respective to each strain, concentrations used in each experiment were as follows: for *B. subtilis* 256 μg/ml, *S. aureus* 64 μg/ml, *K. pneumoniae* 128 μg/ml, *E. coli* 64 μg/ml, *E. cloacae* 128 μg/ml). For this purpose, an overnight culture of each strain (standardized to 0.5 McFarland) in LB medium was diluted with fresh LB medium (1:20). Then, 100 μl of inoculum was added to the wells of a 96-wells microtiter plate (Thermo Scientific, USA) and incubated at 37°C for 12 h. After incubation, the unattached cells were removed by washing the wells with 0.85% NaCl thrice. Bacterial cells attached to the wells were stained for 10 min with 0.1% crystal violet and then eluted after washing with 0.85% NaCl by the addition of 200 μL of 33% glacial acetic acid. Absorbance at 546 nm was measured to quantify the number of cells adhered to the wells of the microtiter plates in the presence and absence of the chloroform extract of turmeric. Negative and positive controls were included using only sterile growth medium and a working solution, respectively.

For the cell attachment assay using a glass surface, bacterial strains were grown in a glass tube containing LB broth with, and without, the chloroform extract of turmeric (at sub-inhibitory concentrations respective to each strain), and incubated at 37°C for 24 h. After incubation, the planktonic cells were gently removed from the tubes. In order to remove attached cells, 0.5 M NaOH was added to each tube and then vortexed. The cells were then suspended in 0.85% NaCl and the absorbance was measured at 546 nm to quantify the number of cells attached to glass surfaces.

Static biofilm formation assay. The effect of a chloroform extract of turmeric on biofilm formation was assessed using modifications of the method of Christensen et al. (1985). A chloroform extract of turmeric (100 μl) dissolved in DMSO and further diluted in LB broth was added to the wells at a sub-inhibitory concentration (respective to each strain). An overnight culture of each strain (standardized to 0.5 McFarland) in LB medium was diluted with fresh LB medium (1:20). Then 100 μl of this culture was added to the wells of a 96-wells microtiter plate (Thermo Scientific, USA) and incubated at 37°C for 48 h, 72 h, 96

Table 2. Minimum inhibitory concentrations (MICs) of strains for the chloroform extract of turmeric.

Strains	MIC value (μg/ml)
B. subtilis	512
S. aureus	128
K. pneumoniae	256
E. coli	128
E. cloacae	256

h and 120 h. Negative and positive controls were included using only a sterile growth medium and a working solution, respectively. After incubation, the growth medium was removed, and the wells were washed three times with saline (0.85% NaCl), then the biofilms were stained with 0.1% crystal violet for 10 min. The excessive dye was removed by washing thrice with 0.85% NaCl, while cell-bound dye was eluted by adding 200 μL of 33% glacial acetic acid. Finally, the absorbance of the eluted solution was measured at 546 nm.

Motility assays. Motility experiments were performed following the method of Rashid and Kornberg (2000), with some modifications in order to assess the effect of sub-inhibitory concentrations of a chloroform extract of turmeric. Briefly, each bacterial strain was grown in LB broth with, and without, a chloroform extract of turmeric (at 0.5 × MIC and 0.25 × MIC concentrations). Then, 10 μl of overnight broth culture of each strain was spotted on the center of swim and swarm plates and incubated at 37°C for 24 h. The degree of swimming and swarming was determined by measuring the diameter of migration of each strain. While, in the case of twitching motility, twitching agar was spotted at the bottom of the petri plate. The ability of bacteria to twitch strongly to the glass surface was examined after 24 h of incubation. The agar was removed and the diameter (mm) of migration was measured after staining with 0.1% crystal violet for 1 min.

Effect of the chloroform extract of turmeric on the hydrophobicity of bacteria. To check cell surface hydrophobicity, the method of Rosenberg et al. (1980) was followed. Briefly, cells were grown in LB broth supplemented with a chloroform extract of turmeric (at sub-inhibitory concentrations with respect to each strain) and without the extract, harvested during the log phase, washed and resuspended in a phosphate buffer (0.1 M, pH = 7) to obtain an optical density of 0.5 at a wavelength of 550 nm (A_0). To 3 ml of cell suspension, 0.4 ml of xylene (hydrocarbon) was added. This suspension was equilibrated by placing the tubes in a water bath at 25°C for 10 min. The tubes were then placed at room temperature for 15 min for phase separation, after which the lower phase (aqueous) was taken and its absorbance was measured at 550 nm (A_1). The results were expressed as the percentage of bacterial adherence to hydrocarbons (A) as compared with the control using the following relationship:

$$A = [(A_0 - A_1)/A_0] \times 100.$$

The bacterial strains were considered to be hydrophilic when the values were <20%, moderately hydrophobic when the values were in the range of 20–50%, and strongly hydrophobic when the values were >50% (Mattos-Guaraldi et al., 1999).

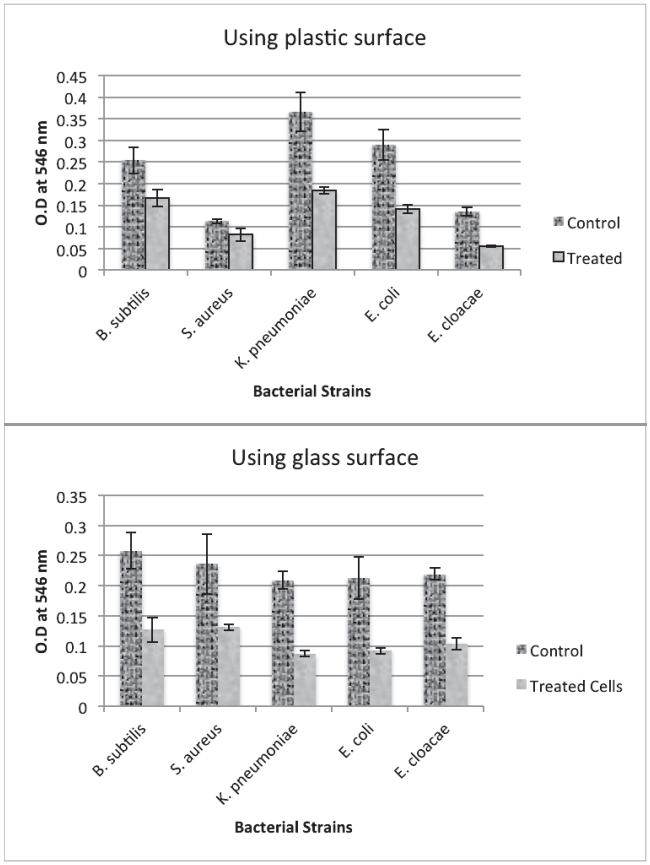

Fig. 2. Bacterial adhesion to polystyrene and glass surfaces in the presence and absence of a sub-inhibitory concentration of the chloroform extract of turmeric for antibiotic resistant bacteria.

In order to check the % inhibition of surface hydrophobicity, the following relationship was used:

% Inhibition of hydrophobicity
= [% hydrophobicity of control
– % hydrophobicity of treated cells
/% hydrophobicity of control] × 100.

Biofilm detachment assay. In order to perform the biofilm detachment experiment, the method of Davies et al. (1998) was followed with some modifications. Briefly, the chloroform extract of turmeric (100 μl) dissolved in DMSO, and further diluted in LB broth, was added to the wells at a sub-inhibitory concentration (respective to each strain). An overnight culture of each strain (standardized to 0.5 McFarland) in LB medium was diluted with fresh LB medium (1:20). Then, 100 μl of this culture was added to the wells of a 96-wells microtiter plate and incubated at 37°C for 48 h, 72 h, 96 h and 120 h. After incubation, 3 μl of sodium dodecyl sulfate (10% w/v) was added to each well, the plate was incubated at 37°C for 30 min. After incubation, the suspended cells were discarded and washed three times with 0.85% NaCl. The bound cells were quantified following the same protocol as described earlier in the static biofilm assay.

EPS extraction. For the production of EPS, the medium

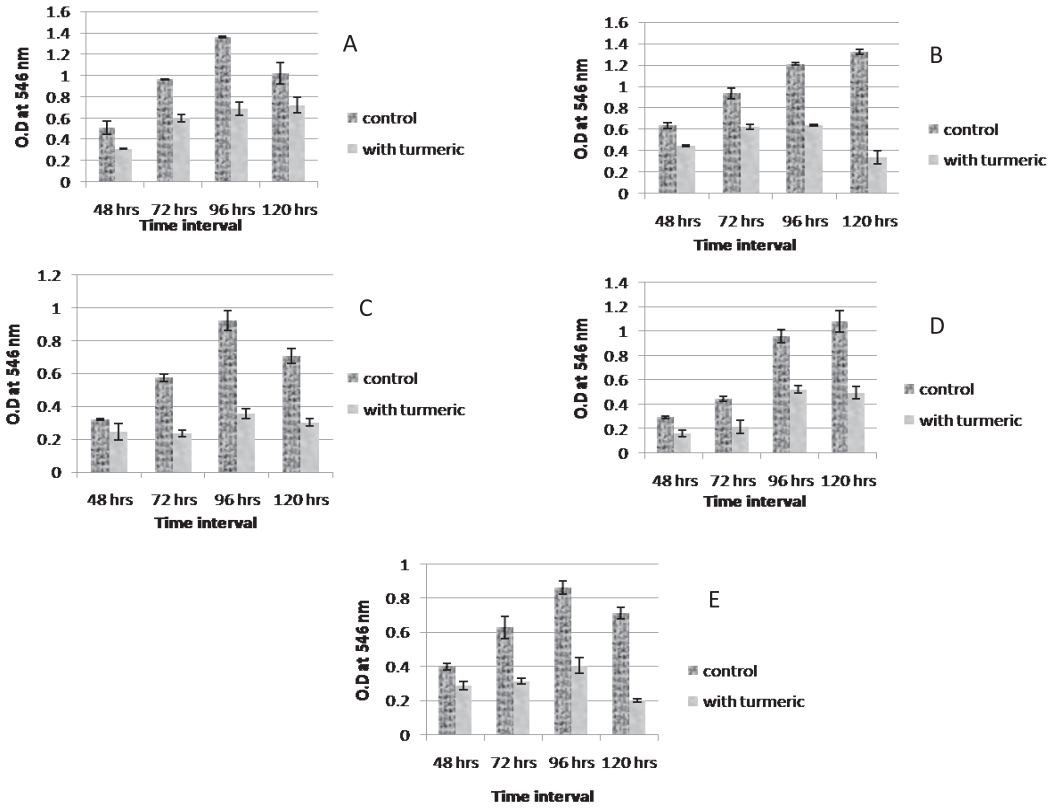

Fig. 3. Quantification of biofilm formation for antibiotic resistant strains.

Cells were grown at 37°C in the presence and absence of a sub-inhibitory concentration of the chloroform extract of turmeric for 48, 72, 96 and 120 h. (a) Biofilm formation by *E. coli*, (b) biofilm formation by *K. pneumoniae*, (c) biofilm formation by *E. cloacae*, (d) biofilm formation by *S. aureus*, (e) biofilm formation by *B. subtilis*.

Table 3. Table showing results of percentage hydrophobicity and inhibition of hydrophobicity in the presence of sub-inhibitory concentration of the chloroform extract of turmeric.

Strains	% age hydrophobicity in the absence of extract	% age hydrophobicity in the presence of extract	Decrease in hydrophobicity (%)
B. subtilis	36.4 ± 1.595	25.8 ± 1.0	29.1 ± 1.760
S. aureus	33.6 ± 1.345	56.4 ± 4.0	–67.8 ± 3.0
K. pneumoniae	60.25 ± 2.0	6.65 ± 1.0	89.9 ± 3.90
E. coli	43.25 ± 4.670	37.5 ± 1.5	13.0 ± 1.20
E. cloacae	38.2 ± 2.10	3.80 ± 0.8	90.05 ± 3.30

reported in Verhoef et al. (2003) was used. Briefly, an overnight culture of each strain (standardized at 0.5 McFarland) was added to 100 ml of medium having a chloroform extract of turmeric at sub-inhibitory concentrations, respective to each strain. The flasks were incubated at 37°C for 48 h on an orbital shaker (160 rpm). After incubation, EPS was extracted following the method of De Vuyst et al. (1998). After centrifugation (10,000 rpm, at 4°C/15 min), the supernatant containing EPS was precipitated by adding three volumes of acetone (pre-chilled). After 48 h, centrifugation (at 15000 rpm at 4°C/15 min) was carried out in order to separate the precipitated EPS, which was then dried at 58°C for 24 h. The wet and dry weight of the extracted EPS was measured. The protein and carbohydrate content of the extracted EPS were quantified following the method of Bradford (1976) and the phenol-sulphuric acid method (Dubois et al., 1956) respectively.

Characterization of EPS. An EPS pellet (2 mg) was mixed with dry potassium bromide (200 milligram), and the mixture was pressed into a 16-mm-diameter mold, which was used for ATR-FTIR (attenuated total reflectance Fourier transform infrared) spectroscopy in the region of 500–4000 cm^{-1} (Mancuso Nichols et al., 2004).

Statistical analysis. The Student's *t*-test was employed to check the significance of each experiment by using Microsoft, Excel. A value of $p < 0.05$ was considered to be statistically significant.

Results

GC-MS profiling of the chloroform extract of turmeric revealed the presence of many compounds particularly sesquiterpenes and fatty acid groups as shown in Fig. 1.

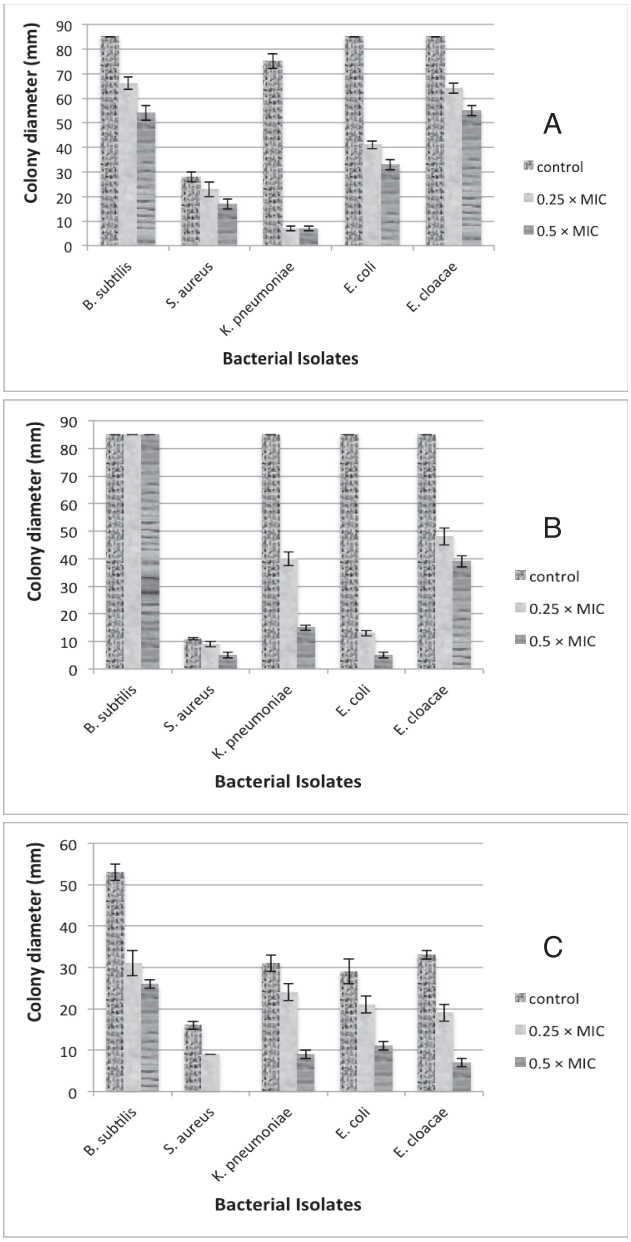

Fig. 4. Quantification of different types of motilities by antibiotic resistant strains in the presence of varying concentrations of the chloroform extract of turmeric (a) Swarming motility, (b) swimming motility, (c) twitching motility.

Antibiotic susceptibility profiling was done by using twelve antibiotics belonging to different groups according to their mode of actions. It was observed that all of the tested strains were susceptible to chloramphenicol. Antibiotic resistant profiling had indicated that all of the strains exhibited resistance against a number of antibiotics tested as shown in Table 1. For example, it was observed that *S. aureus* exhibited resistance against all tested antibiotics except for chloramphenicol and erythromycin. Similarly *E. coli* exhibited susceptibility only towards ertapenum and chloramphenicol.

In order to evaluate the antibacterial effect of the chloroform extract of turmeric, minimum inhibitory concentrations (MICs) were determined for bacterial strains used in the present study. The results indicated that MIC values ranged between 128–512 μg/ml against the tested strains (Table 2).

Cell attachment assay by using the polystyrene surfaces of 96-wells microtiter plates revealed that, after 12 h of incubation in the presence of the extract, bacterial attachment to the 96-wells microtiter plate surfaces was significantly reduced ($p < 0.05$) as compared with the control cells. Inhibition of adherence by the chloroform extract was found to be 38.9–60.2%. Maximum anti-adhesion potential was observed in the case of *K. pneumoniae* (% age inhibition of adherence was found to be 60.2%) followed by *E. cloacae* as shown in Fig. 2. Similarly, the results of bacterial attachment to the glass surface also revealed a significant reduction of cell adherence as compared with untreated cells. The inhibition of adherence by the chloroform extract of turmeric was found to be 44.5–58.3%. Maximum anti-adhesion potential was observed in the case of the *K. pneumoniae* (% age inhibition of adherence was found to be 58.3%) followed by *E. coli* as indicated in Fig. 2.

In order to detect the antibiofilm activity of the chloroform extract of turmeric, biofilm formation was checked at different time intervals, i.e. 48 h, 72 h, 96 h and 120 h. It was observed that the chloroform extract resulted in 23–74.5% reduction of biofilm formation in treated cells as compared with the controls (cultures without chloroform extract). Maximum inhibition of biofilm formation was observed in the case of *K. pneumoniae* followed by *B. subtilis*. In most of the cases, maximum biofilm formation was observed after 96 h while, in the case of *S. aureus* and *K. pneumoniae*, biofilm formation became a maximum at 120 h of incubation. The targeted strains also revealed significant differences ($p < 0.05$) in their biofilm-forming ability as indicated by differences in absorbance measured at 546 nm (Fig. 3).

The results of motility assays indicated that the chloroform extract of turmeric inhibited the swimming, swarming and twitching motilities of bacteria in a dose-dependent manner as observed by a decrease in the diameter of the distance travelled by the isolates in the presence of varying concentrations of the chloroform extract (Figs. 4 and 5). It was observed that with an increase in the concentration of the chloroform extract the level of each type of motility was significantly reduced ($p < 0.05$). Moreover, it was elucidated from the results that the repression of each type of motility was more pronounced in the case of Gram negative bacteria as compared with Gram positive bacteria as shown in Fig. 4.

Our data indicated that all of the strains studied here showed hydrophobic behavior (most of the strains were moderately hydrophobic); however, *K. pneumoniae* exhibited a strong hydrophobic nature as indicated by a hydrophobicity of 60.25%. It was observed that, in general, there was a statistically significant ($p < 0.05$) change in the hydrophobic behavior of targeted bacteria treated with the chloroform extract of turmeric (at sub-inhibitory concentrations) as shown in Table 3. Our results indicated that *E. cloacae* and *K. pneumoniae* exhibited hydrophilic behavior after treatment with the chloroform extract. While in the case of *S. aureus*, there was an increase in hydrophobic behavior (upto 67%) in the presence of the extract as compared with the control.

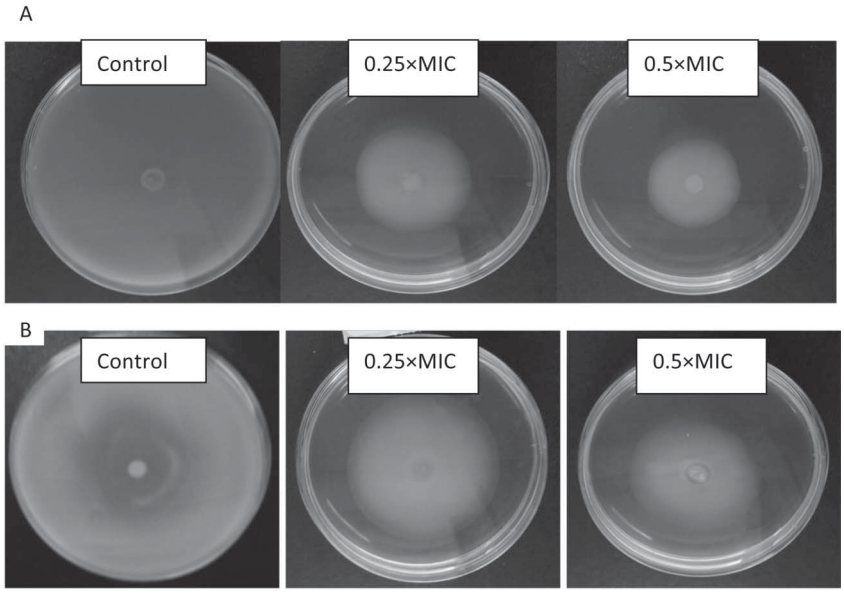

Fig. 5. Dose-dependent inhibition of swimming and swarming motilities by the chloroform extract of turmeric.

Cells were inoculated at the center of an agar media containing chloroform extract of turmeric at various concentrations. (a) Swimming motility in *E. coli*, (b) swarming motility in *E. cloacae*.

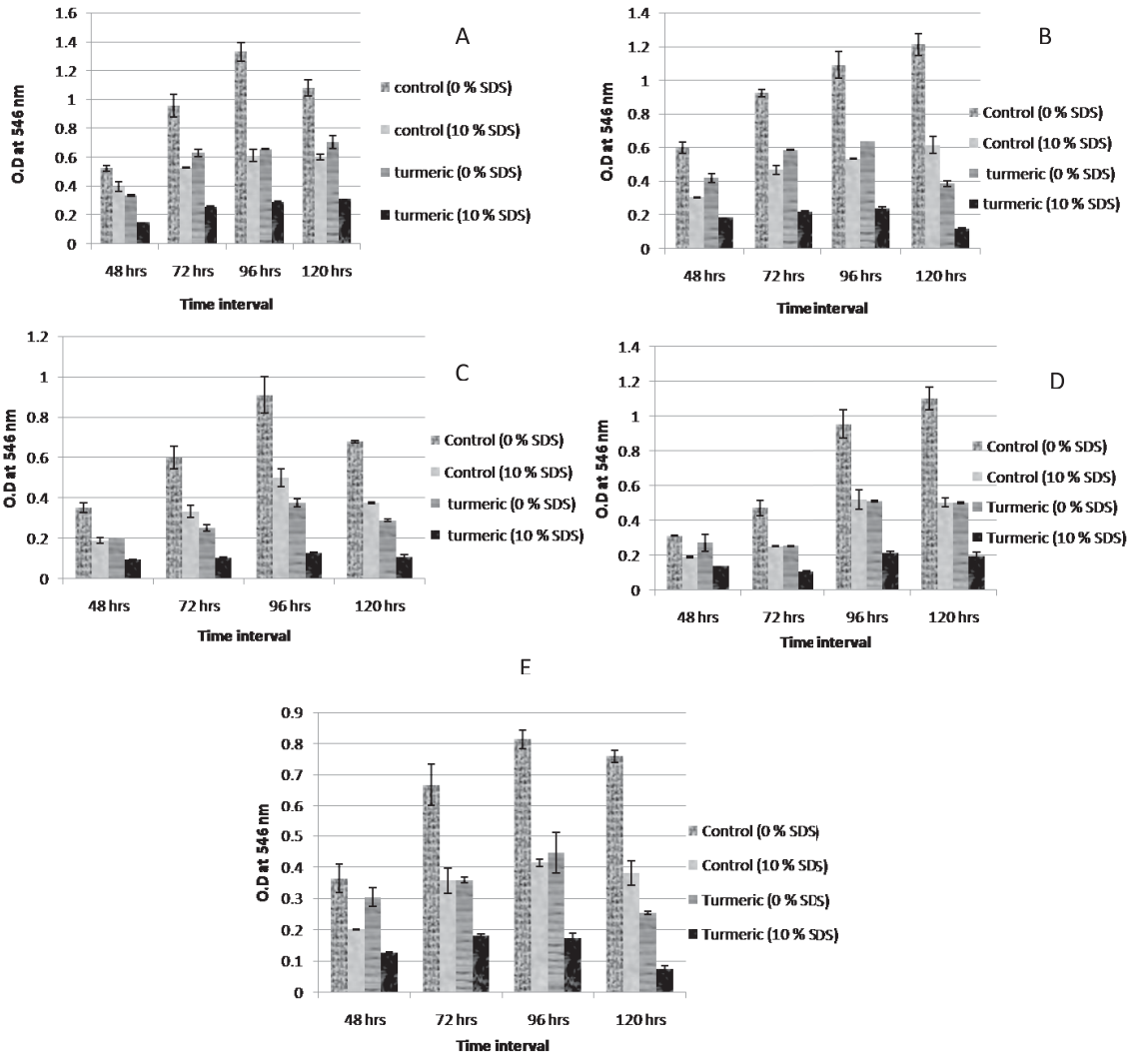

Fig. 6. SDS detachment of biofilms formed by antibiotic resistant strains in the presence and absence of the chloroform extract of turmeric. Error bars indicate a standard error of mean for three measurements.

Table 4. Wet and dry weight of EPS (mg/100 ml of culture) extracted from antibiotic resistant bacteria in the presence of sub-inhibitory concentrations of the chloroform extract of turmeric.

Strains	Control		Treated cells	
	Wet weight	Dry weight	Wet weight	Dry weight
B. subtilis	238 ± 4	106 ± 2	126.5 ± 3.5	53.5 ± 2.5
E. coli	224 ± 1	111.5 ± 2	101 ± 1.5	56.5 ± 0.5
E. cloacae	217 ± 3	136 ± 2	119 ± 1	54.5 ± 2.5
K. pneumoniae	398 ± 4	150.5 ± 2.5	172 ± 1	78 ± 0.5
S. aureus	124 ± 4	62 ± 2	79.5 ± 4.5	42.5 ± 1

Table 5. Carbohydrate and protein content of EPS (μg/g of EPS) extracted from antibiotic resistant bacteria in the presence of sub-inhibitory concentrations of the chloroform extract of turmeric.

Bacterial strains	Carbohydrate content (μg/g of EPS)		Protein content (μg/g of EPS)	
	Untreated cells	Treated cells	Untreated cells	Treated cells
B. subtilis	256.5 ± 1.5	151.5 ± 3.5	194.5 ± 4.5	109.5 ± 2.5
E. coli	475.5 ± 6.5	206.5 ± 2.5	214.5 ± 1.5	65.5 ± 2.5
E. cloacae	399.5 ± 1.5	224.5 ± 2.5	226.5 ± 1.5	55.8 ± 4.5
K. pneumoniae	516.5 ± 4.5	209.5 ± 2.5	239.5 ± 2.5	107.5 ± 0.5
S. aureus	336 ± 1.5	184.5 ± 4.5	175.5 ± 2.5	105.5 ± 1.5

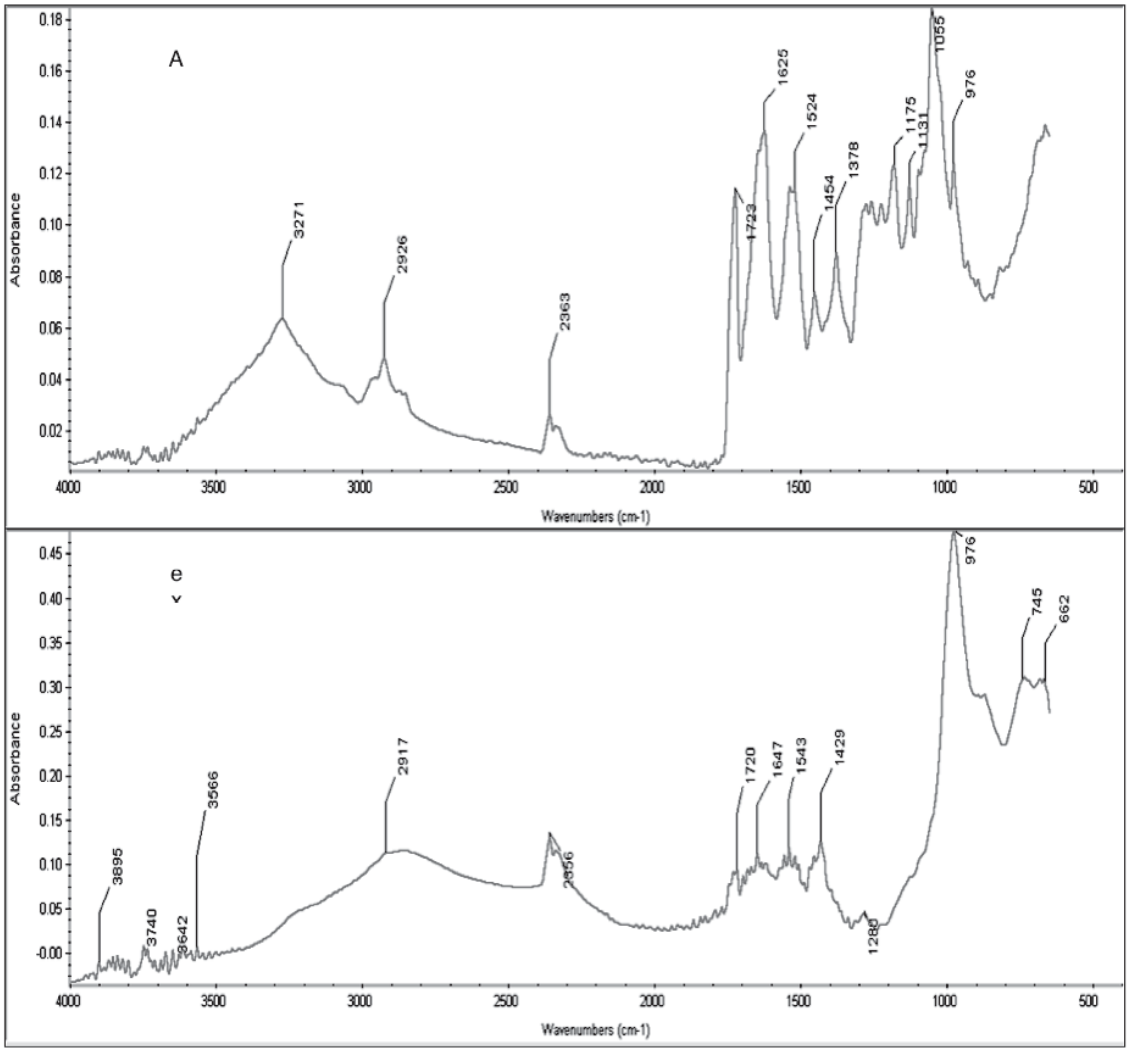

Fig. 7. (a) ATR-FTIR spectra of EPS of cells of *K. pneumoniae* grown in the absence of the chloroform extract of turmeric; (b) ATR-FTIR spectra of EPS of cells of *K. pneumoniae* grown in the presence of the chloroform extract.

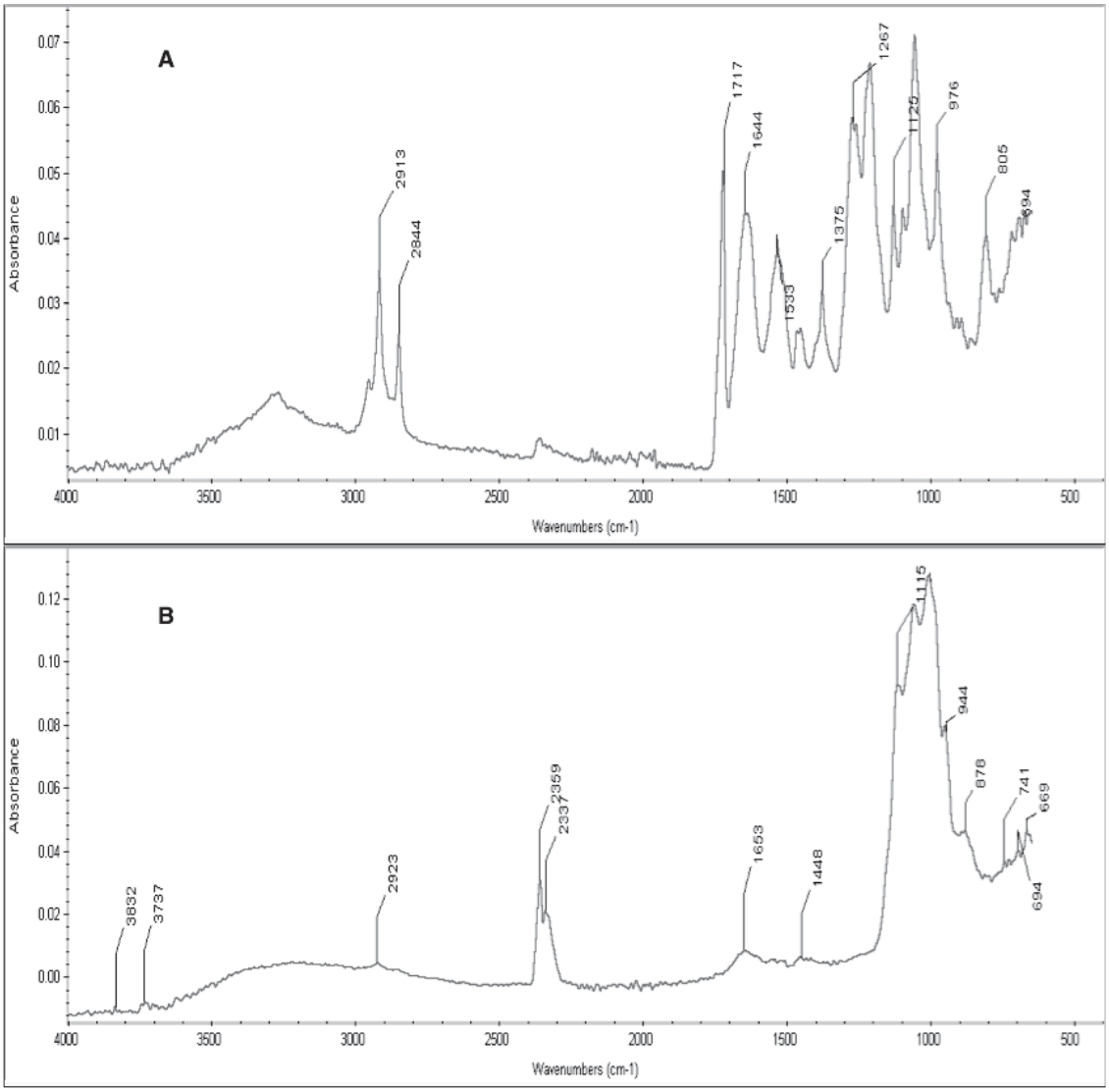

Fig. 8. (a) ATR-FTIR spectra of EPS of cells of *E. coli* grown in the absence of the chloroform extract; (b) ATR-FTIR spectra of EPS of cells of *E. coli* grown in the presence of the chloroform extract.

In order to evaluate the effect of the chloroform extract of turmeric on the detachment efficiency of sodium dodecyl sulfate (SDS), biofilms formed on the surfaces of microtiter plates in the presence and absence of the chloroform extract were quantified after the addition of SDS. The results indicated that the presence of the chloroform extract increased the detachment efficiency of SDS as shown in Fig. 6. It was observed that the detachment efficiency of the surfactant was higher in the case of biofilms formed in the presence of the chloroform extract (ranging from 49.1–71.22%). While, in the case of the control cells, the detachment of biofilms by SDS ranged from 24.13–54.36% (Fig. 6).

The results indicated that the wet weight of EPS extracted from the control cells was in the range of 124–398 mg/100 ml of culture, while the dry weight of EPS for the control cells was in the range of 62–150.5 mg/100 ml of culture. Maximum EPS was extracted from the cells of *K. pneumoniae*, while the minimum was obtained from *S. aureus*. The results revealed that the treatment of cells with chloroform extract resulted in a decrease in the wet and dry weight of the extracted EPS. It was observed that there was a 35.8–56.8% decrease in the wet weight of extracted EPS in the presence of chloroform extract, while the dry weight of EPS was reduced in the range of 31.5–59.9% (Table 4). The maximum decrease in the extracted EPS was observed for *K. pneumoniae* (as indicated from wet and dry weight analysis) as shown in Table 4.

The quantitative estimation of the protein and carbohydrate contents of extracted EPS revealed that the treatment of bacterial cells with the chloroform extract of turmeric resulted in significantly reduction in the protein and carbohydrate contents of EPS, as compared with EPS extracted from untreated cells. As shown in Table 5, there was a 40.94–59.4% reduction in the estimated carbohydrate content, whereas the inhibition of protein content was found to be 39.88–75.4%.

FTIR spectroscopy was carried out to analyze the differences in spectra and functional groups in the EPS of cells treated with, and without, chloroform extract of turmeric. The results indicated a corresponding loss of vital functional groups from the EPS of cells treated with chloroform extract as compared with untreated cells. The IR spectrum of *K. pneumoniae* revealed the presence of -OH

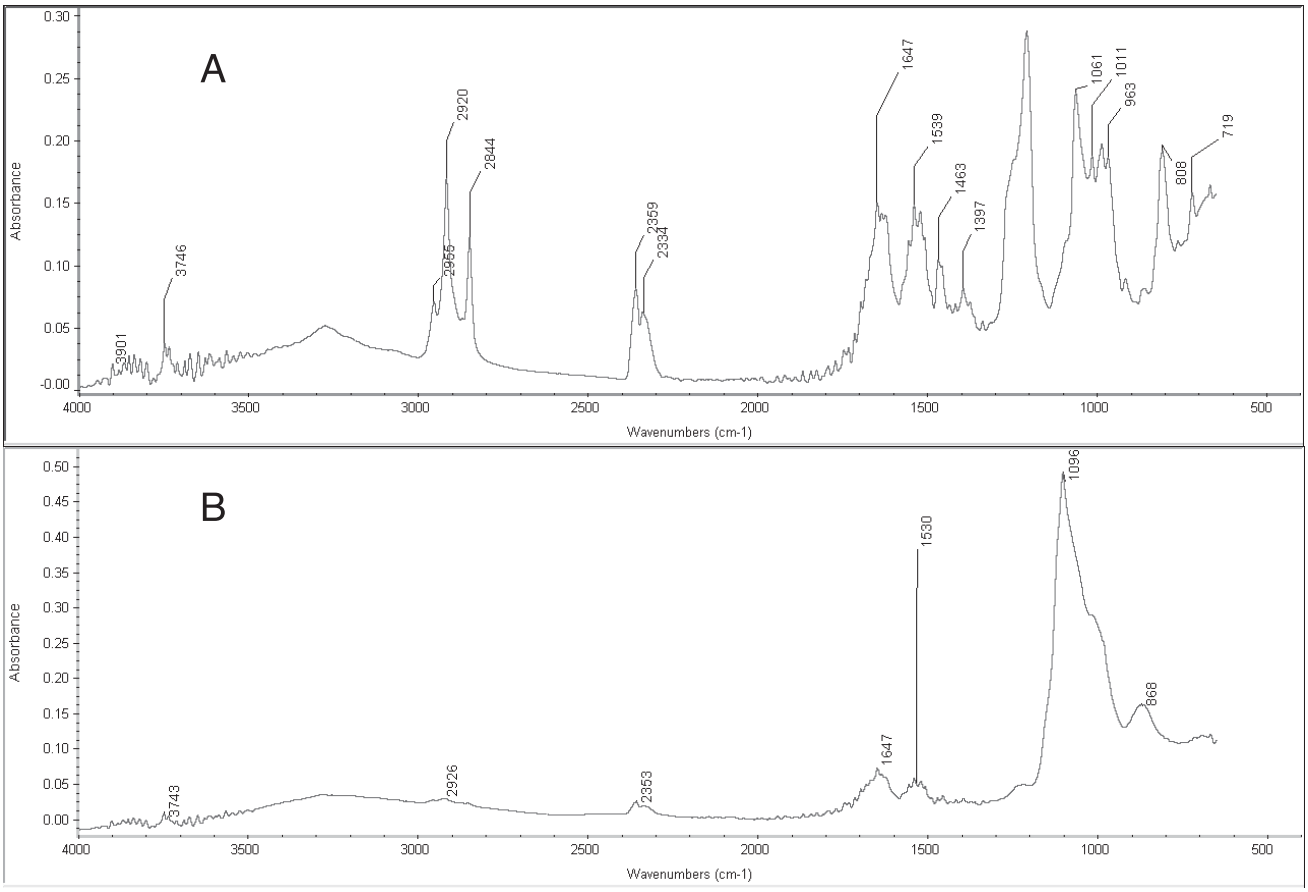

Fig. 9. (a) ATR-FTIR spectra of EPS of cells of *E. cloacae* grown in the absence of the chloroform extract; (b) ATR-FTIR spectra of EPS of cells of *E. cloacae* grown in the presence of the chloroform extract.

group into polymeric compounds at 3271 cm^{-1}. The band at 1723 cm^{-1} corresponds to a stretching vibration of C=O of esters. In addition, the bands appearing at 1625 cm^{-1} and 1524 cm^{-1} were characteristics of amide I and amide II of proteins, respectively. The band appearing at 1055 cm^{-1} revealed the presence of an OH group of polysaccharides and their derivatives. While the band at 976 cm^{-1} was associated with stretching vibrations of nucleic acid (Fig. 7a). The IR spectrum of EPS extracted from cells treated with chloroform extract exhibited reduced band intensities in the region of 1400–1800 cm^{-1} indicating a loss of functional groups related to polysaccharides and proteins (Fig. 7b). A similar trend was observed for all other strains indicating that the protein and polysaccharides content of EPS was reduced because of the corresponding loss of functional groups belonging to these compounds after treatment of the cells with a chloroform extract of turmeric (Figs. 8b–11b) compared with IR spectra of EPS extracted from cells grown in the absence of a chloroform extract of turmeric (Figs. 8a–11a).

Discussion

The findings of our study have indicated the potential use of the chloroform extract of turmeric against biofilm formers exhibiting drug resistance. The present study has demonstrated that a chloroform extract of turmeric contains a variety of non-polar phytochemicals, particularly sesquiterpenes, turmerone, and fatty acids as indicated by GC-MS analysis, that might be responsible for antibiofilm activities. A number of studies have revealed that natural products isolated from plants can be effectively used as biofilm inhibitors because of their low toxicity and increased specificity (Koo and Jeon, 2009).

From the MIC data obtained, it was observed that the chloroform extract was effective against both Gram positive, as well as Gram negative, bacteria. Sub-inhibitory concentrations of turmeric extract were used to determine the effect on adherence, different types of motilities, EPS production, and cell surface hydrophobicity. The sub-inhibitory concentrations of antibacterial agents, such as antibiotics, had a remarkable effect on the bacterial characteristics. One of the previous studies had shown that significant changes were observed in *Pseudomonas aeruginosa* treated with sub-inhibitory concentrations of piperacillin/tazobactum, such as a decrease in adherence and biofilm formation, an increased sensitivity to oxidative stress, and a lowered swimming and twitching motility (Fonseca et al., 2004). Our results have shown that this concentration effectively reduced adherence, cell surface hydrophobicity, EPS production, and the colony diameter of bacterial strains, while studying different types of motilities.

It is an established fact that adhesion is the initial step in the complex process of biofilm formation. Therefore, targeting this step will decrease the potential of bacterial cells to form biofilms and, ultimately, its virulence. In our study, significant inhibition of cell attachment to differ-

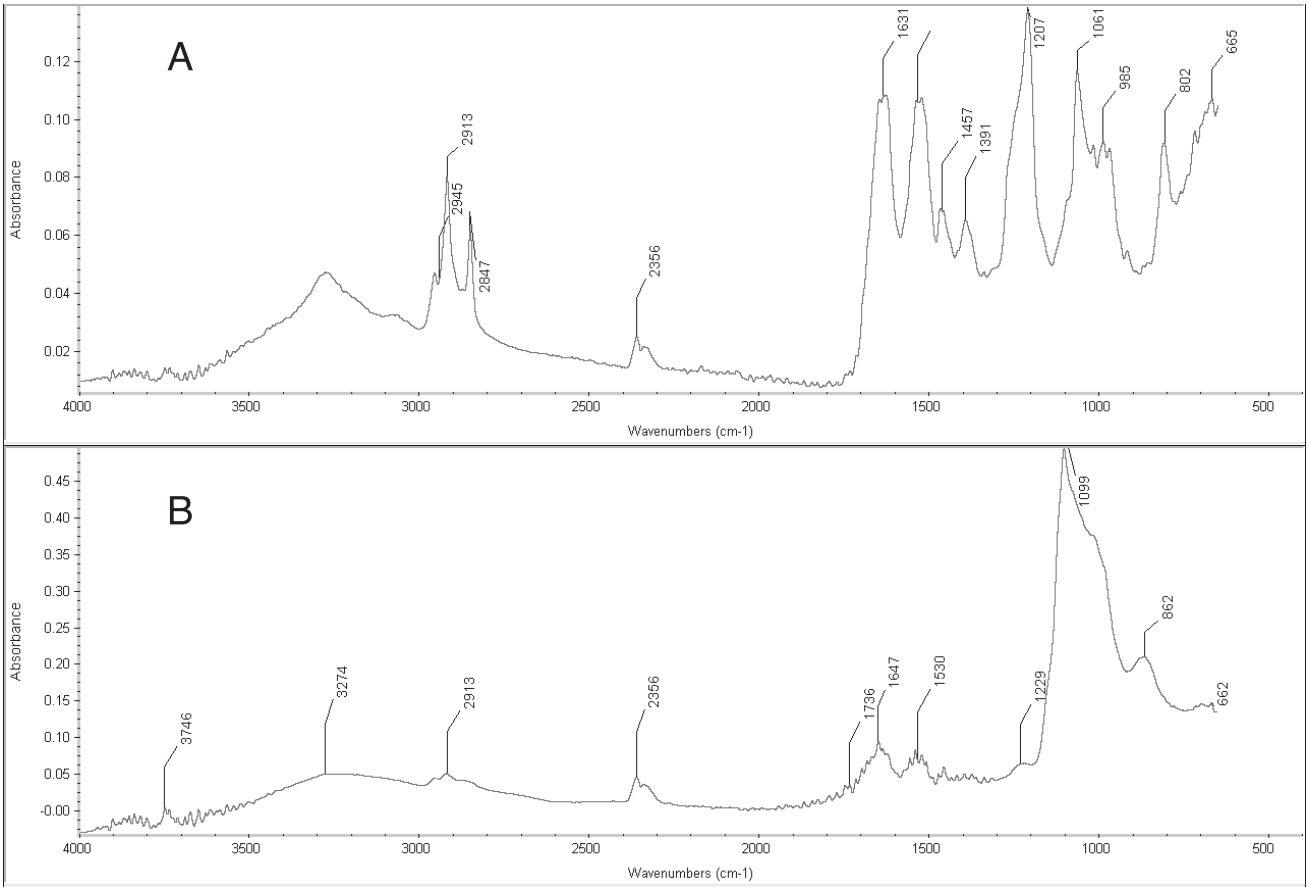

Fig. 10. (a) ATR-FTIR spectra of EPS of cells of *S. aureus* grown in the absence of the chloroform extract; (b) ATR-FTIR spectra of EPS of cells of *S. aureus* grown in the presence of the chloroform extract.

ent surfaces, such as plastic and glass, was observed after treatment of cells with a chloroform extract of turmeric, indicating the involvement of some other factors besides cell surface hydrophobicity. It has been reported that adhesion of microorganisms to various surfaces also involves a physico-chemical process that can efficiently mask the role of cell surface hydrophobicity in adhesion. Numerous studies have explored the belief of the absence of a direct relationship between adhesion to hydrophobic/hydrophilic surfaces and cell surface hydrophobicity in a number of pathogens such as *S. epidermidis* (Cerca et al., 2005) and *S. aureus* (Zmantar et al., 2011).

Moreover, a critical element contributing to reduced adherence could be the destruction of an EPS matrix of bacteria by the chloroform extract. Previous studies had indicated the role of EPS in altering the physicochemical properties of cell surfaces, such as the zeta potential and cell surface hydrophobicity, thus affecting bacterial attachment and ultimate colonization (Aslim et al., 2007; Deepika et al., 2009). Another element contributing to this decrease in cell attachment might be the inhibition of flagella-directed motility. As indicated in our study, chloroform extracts of turmeric significantly inhibited flagella-directed swarming and swimming motility as compared with control cells. Furthermore, pilus-directed twitching motility was also decreased in the presence of a chloroform extract of turmeric. Previous studies also indicated the role of flagellum-directed motility in early attachment during the biofilm formation of bacteria by combating the interfacial forces of repulsion (Kalmokoff et al., 2006; O'Toole and Kolter, 1998).

It has been observed that one of the critical factors involved in the bacterial adherence to host tissues includes cell surface hydrophobicity. The hydrophobic features of cell surfaces are important for the adhesion of cells to biotic or abiotic surfaces and to penetrate inside the host tissues (Goulter et al., 2009; Heilmann, 2011; Rodrigues and Elimelech, 2009). In our study, when bacteria were incubated in the presence of sub-inhibitory concentrations of a chloroform extract of turmeric, there was a significant difference in the surface properties of bacteria. It was observed that cells such as *K. pneumoniae* and *E. cloacae* that were initially hydrophobic became hydrophilic after treatment with plant extracts. This decrease in cell surface hydrophobicity can be attributed to the presence of different compounds in the chloroform extract, particularly amphipathic compounds that might have the potential to block the hydrophobic sites, thus decreasing the cell surface hydrophobicity. As indicated in a study by Barness et al. (1988), the presence of amphipathic compounds, such as serratamolide, decreased the cell surface hydrophobicity of *Serratia marcescens*. Previous studies had also indicated the changes in hydrophobic behavior of *Streptococcus sanguinis*, *Streptococcus mitis* and *Actinomyces* sp. after treatment with *Psidium guajava* (Razak et al., 2006). Hasan et al. (2015) also observed that ginger extract significantly inhibited the hydrophobicity and cell adherence of *S. mutans*. However, in contrast to all other

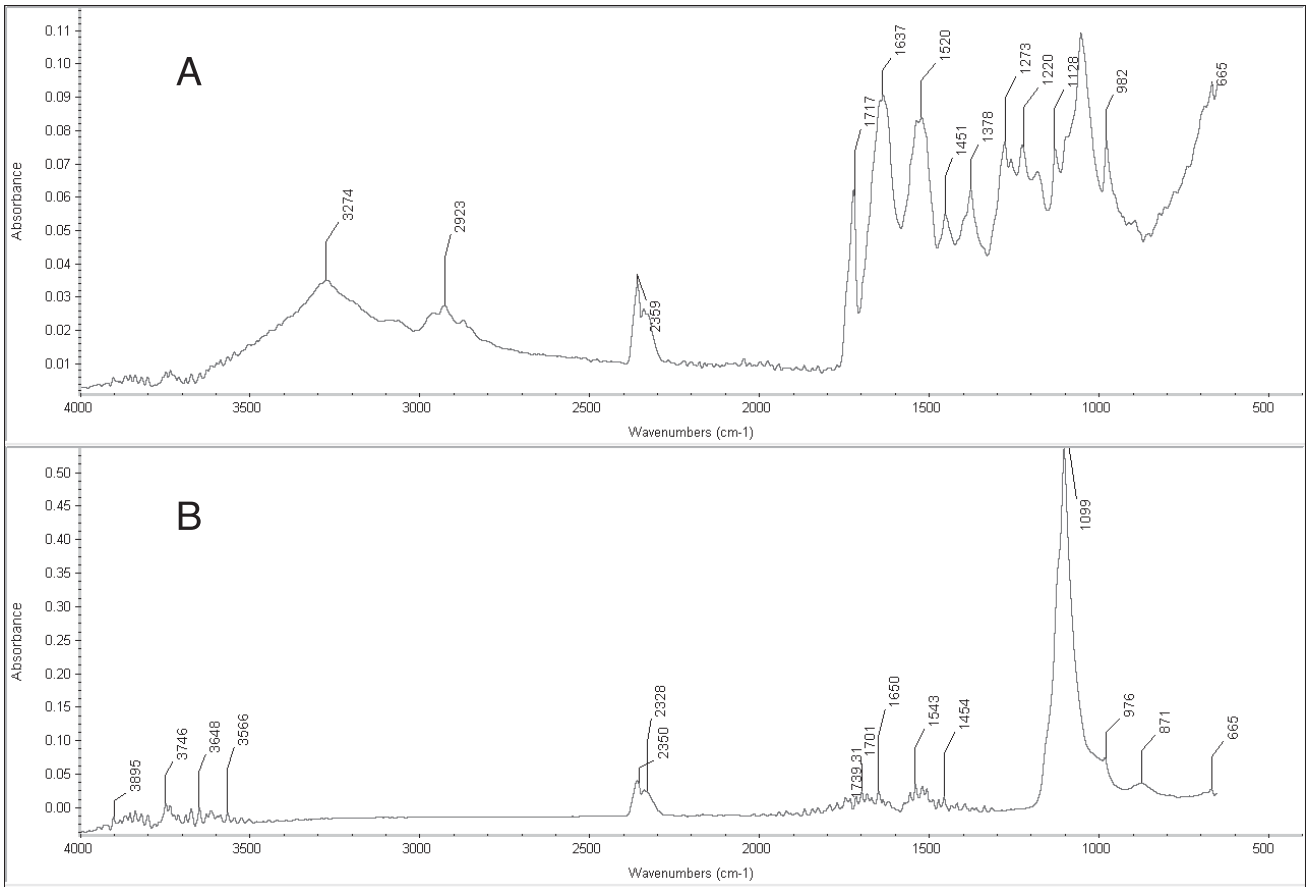

Fig. 11. (a) ATR-FTIR spectra of EPS of cells of *B. subtilis* grown in the absence of the chloroform extract; (b) ATR-FTIR spectra of EPS of cells of *B. subtilis* grown in the presence of the chloroform extract.

tested bacterial strains, there was an increase in cell surface hydrophobicity in *S. aureus* after the addition of a chloroform extract of turmeric, indicating that cell surface hydrophobicity is not the only key element contributing towards initial cell attachment and ultimate biofilm formation. It has been observed that extracellular polysaccharides secreted by different species of *Staphylococcus* are essential for adhesion to numerous surfaces, as well as for cell-cell attachment (Joyce et al., 2003). As indicated in the present study, the addition of chloroform extract resulted in a decline in the carbohydrate and protein contents of EPS in *S. aureus* and ultimately reduced the adherence and biofilm-forming capacity of *S. aureus* by destroying the EPS bridge.

The results of the biofilm detachment assay indicated that a chloroform extract of turmeric could enhance the potential of surfactants such as SDS to remove biofilms from plastic surfaces. These results suggested that biofilms formed in the presence of a chloroform extract were loosely attached to microtiter plate surfaces and removed easily by the treatment of the surfactant, as compared with biofilms formed in the absence of chloroform extracts. This potential of biofilm inhibitors can be used in industries to remove biofilms formed on membrane filters during the treatment of water (Kappachery et al., 2010).

EPS production by microorganisms is generally considered to play a vital role during the process of biofilm formation and cell-to-cell adhesion. EPS acts as a bridge for adhering negatively charged bacterial cells to positively and negatively charged surfaces (Azeredo and Oliveira, 2000). Our study has indicated that the treatment of the cells with chloroform extract resulted in the loss of some vital functional groups belonging to major constituents (such as polysaccharides and proteins) of this bridge, thus reducing the potential of bacterial cells to form biofilms, cell surface hydrophobicity, and cell-to-cell adhesion.

Medicinal plants exhibit some important biological activities, mainly due to the presence of vital phytochemical constituents. Our study revealed the presence of many phytochemicals particularly sesquiterpenes, turmerone, and fatty acid compounds in a chloroform extract. Previous studies had indicated that fatty acid compounds in nonpolar extracts of plants were responsible for antimicrobial, antioxidant, anti-inflammatory, and antiproliferative activities (Geetha et al., 2013; Selvamangai and Bhaskar, 2012). Many scientists have proved that essential oils of turmeric are responsible for antimicrobial activities (Dhingra et al., 2007; Garcia et al., 2008; Pawar and Thaker, 2007).

In short, the present study has clearly indicated the potential use of a chloroform extract of turmeric in clinical settings, as it significantly affected the attributes necessary for the initial attachment and ultimate biofilm formation of pathogens exhibiting drug resistance. This study provides the baseline for future research in exploring bioactive compounds in turmeric mainly responsible to target the necessary attributes of biofilm formation.

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Potential Conflict of Interest

None declared.

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