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Effect of tobacco tar on *Staphylococcus aureus and Candida albicans* biofilm formation

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Twenty compounds were determined in the tar of cigarette smoke. The tar was obtained using tobacco filters and then analyzed by liquid chromatography-mass spectrometry (LC-MS). Tobacco tar expressed marked decrease in the susceptibility of *Staphylococcus aureus* to penicillin, tetracycline and amoxicillin/clavulanic antibiotics and showed marked decrease in susceptibility of *Candida albicans* to the tested antifungals except for clotrimazole and ketoconazole. Tobacco tar decreased the permeability of the tested organisms to ethidium bromide in the presence of antimicrobials, after 2 h for both *S. aureus* (17.8-20%) and *C. albicans* (13.3-16.3%) and decreased the adherence of the tested microorganisms at concentrations above Minimal Inhibitory Concentration (MIC). Tobacco tar showed a marked increase in the hydrophobicity of the tested microorganisms by 2.5 to 7 fold. Tobacco tar increased or upregulated *ALS1* and *HWP1* genes that play an important role in adhesion, hyphae formation and biofilm formation of *C. albicans* and increased the expression of *ica A* gene that regulate biofilm formation of *S. aureus*.

Key words: Tobacco tar, *Staphylococcus aureus, Candida albicans*, antimicrobial susceptibility, ethidium bromide, biofilm formation.

INTRODUCTION

Cigarette smoking is considered as an important global health threat. Despite the repeated and continuous warning from different health organizations all over the world, rates of smoking remain high worldwide (Centers for Disease Control and Prevention, 2011; Warren et al., 2008). Smoking causes a tremendous health burden; not only in smokers but in those exposed to cigarette smoke as well. As CS contains many bioactive compounds, including oxidant, genotoxic, and immunomodulatory factors (Church and Pryor, 1985; Pryor et al., 1998; Feng et al. 2011; Huvenne et al. 2011; Yageta et al. 2011; Ebbert et al., 2007). Cigarette smoke (CS) can result in severe harms for human health such as carcinogenesis (Stampfli and Anderson, 2009), promotion of atherosclerosis (Prasad et al., 2009), and chronic lung disease such as chronic obstructive pulmonary disease

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(COPD), increasing the risk of several infectious diseases (Yao and Rahman, 2011), which may be attributed to the direct action of cigarette smoke (CS) components on human cells.

It was found that chronic obstructive pulmonary disease (COPD) progression is associated with the accumulation of inflammatory mucous exudates in the lumen of the lung, and infiltration of the wall by innate and adaptive inflammatory immune cells; these changes are coupled to a repair and remodeling process that ultimately thickens the airways walls (Hogg et al., 2004). Immune response triggered by tobacco smoke components leads to an inflammatory cellular infiltrate and to a pronounced and chronic lung inflammation which was found to be a cause of other pathological changes, including chronic obstructive bronchitis with fibrosis and obstruction of small airways, emphysema with enlargement of airspaces and destruction of lung parenchyma, loss of lung elasticity, and closure of small airways (Barnes, 2004; Cosı´o et al., 2009). Tobacco smoke also leads to lung infections by pathogenic bacteria, fungi and viruses (Acinetobacter, Bacillus, Burkholderia, Clostridium, Klebsiella, *Pseudomonas aeruginosa*, Serratia, Campylobacter, Enterococcus, Proteus, Staphylococcus, and Candida). An additional consequence of long-term smoking is the persistent colonization of the lower respiratory tract by opportunistic pathogens, which often has an amplification effect on and contributes to the progression of the disease (Mizgerd, 2008; Sethi and Murphy, 2001; Kulkarni et al., 2010), particularly during the stage for overgrowth and invasion. Because the colonizing microbiota inhabits human mucosal spaces, microbes may share exposure to a variety of environmental stimuli, including CS. It is thought that exposure of *S. aureus* to CS might induce pathways relevant to both survival and pathogenesis. Also, it facilitates adherence and biofilm formation (Heilmann, 2011).

Since pathogenic bacteria such as *Staphylococcus aureus* and fungi such as *Candida albicans* are members of the normal microbiota and reside in close proximity to human nasopharyngeal cells, we thought that bioactive components of CS might affect these organisms and potentiate their virulence. *S. aureus* is a Gram-positive pathogen that colonizes the skin and mucosal spaces of human hosts (Lowy, 1998; Murphy et al., 2009), with a population-wide carriage rate estimated at 20 to 32% (Shopsin et al., 2000; Wertheim et al., 2005). It is considered as one of the important causes of community- and hospital-acquired infections that ranges in severity from uncomplicated cellulites to deep-seated infections such as endocarditis and osteomyelitis (Boucher et al., 2010). *C. albicans* is the most common opportunistic microorganism that is a part of the normal microbial flora found on mucosal surfaces such as those of the oral cavity, gastrointestinal tract, and vagina in human beings and domestic animals (Richardson and Lass-Florl, 2008). This yeast is known to be the commonly cause of mucosal and invasive fungal infections observed in humans (Morschhauser, 2010). Host protection against *C. albicans* infection is complex and includes different subsets of the immune defense system (Rouabhia et al., 2002; Rouabhia et al., 2005; Saunus et al., 2008). Several factors may increase the risk of being infected by *C. albicans* such as long-term antibiotics intake, leukemia, AIDS, malignancy, radiation therapy for head and neck cancer, or any other factors that affect the immune system (Nave and Mueller, 2013; Pinel et al., 2013; Fidel, 2011). In addition, smoking is one of the environmental factors that can promote *Candida* infections (Baboni et al., 2009, Soysa and Ellepola, 2005).

The aim of this study was to determine the components of the tar of cigarette smoke by LC-MS analysis, in addition to investigation of the effect of tobacco tar on *S. aureus* and *C. albicans* adhesion, growth, and biofilm formation, and on the activation of several genes involved in the virulence of these microorganisms.

MATERIALS AND METHODS

Extraction of tobacco tar

The tar of tobacco was obtanied from LM blue cigarettes (light, tar: 6 mg and nicotine: 0.5 mg, Philip Morris Misr Co., Egypt) by means of smokers throught 76 tobacco fiters (Friend Holder Mini, Japan). The filters reduce the tar and nicotine by double filtering system through high polymer fibre. Each filter contained the tar of 20 cigarettes . The average weight of tar in one filter was 0.0704 g, while the total weight of tar in filters was 5.3504 g. The tar was extracted from filters by methanol.

Liquid chromatography - mass spectrometry (LC/MS) analysis

The methanolic extract of tobacco tar was subjected to LC/MS analysis on Waters ACQITY UPLC coupled to a waters synapt G_2 mass spectrometer (waters Co., USA), which was equipped with an electrospray interface (ESI) with lock spray capability. The system was controlled under Mass Lynx $V_{4.1}$ software (waters Co., USA). The column used was BEH C18 (2.1×50 mm). The analysis was carried out at a temperature of 30°C and the sample was injected as 5 µl at 10°C. The flow rate was 0.3 ml/min. The eluent was 0.1% formic acid in acetonitrile and the elution was carried out by linear solvent strength within 7 min from 0 to 100%, then isocratic with 100% for 1 min, then composition change to 0% within 0.1 min and finally isocratic with 0% within 2 min. The total run was 10.1 min. The identification of the tobacco tar components was carried out by direct comparison of retention times and pattern of fragmentation of each of the identified compounds with those of reference compounds.

Determination of the minimum inhibitory concentration (MIC)

Microorganisms (0.5 ml) of 1×10^6 CFU/ml of 0.5 Mcfarland turbidity were plated in sterile petri dishes, then 20 ml of sterile, molten and cooled (45°C) Muller Hinton agar media was added to all petri dishes. The plates then were rotated slowly to ensure uniform distribution of the microorganisms and then four circular wells of 10 mm diameter were punched using sterile cork borer after agar

solidification. Two fold serial dilutions were performed for tobacco tar starting from a concentration of 5 mg/ml. Equal volumes of each dilution were applied separately to each well in three replicates using a micropipette. Methanol was used as control. All plates were incubated overnight at 37°C, then, zones of inhibition that developed were measured and the average was calculated. MIC was obtained by plotting the natural logarithm of the concentration of extract against the square of inhibition zones. A regression line was drawn through the points. The antilogarithm of the intercept on the logarithm of concentration axis gave the MIC value (Ogbulie et al., 2007; Delahaye et al., 2009).

Adherence assay method (Tissue culture plate method, TCP)

The TCP assay is considered as standard test for detection of biofilm formation and ability of microorganisms to adhere to plastic surface. All isolates were screened for their ability to adhere to the surface of tissue culture plate surface by the TCP method as described by Christensen et al. (1985) with a modification in duration of incubation which was extended to 24 h, according to O'Toole and Kolter (1998).

Effect of tobacco tar on the adherence of the tested strains on plastic surfaces

All strains were first streaked onto Trypticase soy agar and then incubated at 25°C for 48 h. One loopful of the cultured cells were transferred into tubes of trypticase soy broth (TSB) (Difco Laboratories) containing 0.9% D-glucose. After incubation at 25°C for 24 h, the cells were centrifuged and washed twice with 0.5 ml PBS (phosphate buffered saline), followed by vortexing and centrifugation at 5000 g for 5 min. The washed cells were suspended in 1 ml TSB broth and adjusted to a final OD 600 nm value of 1.0 with TSB broth then, used to grow biofilms. One hundred µl of the microbial suspension (OD600) was transferred into the wells of polystyrene 96-well plates (flat bottom; Nunc). TSB broth was used as a negative control. The plates were incubated at 25°C for 90 min (adhesion period). Supernatants including planktonic cells were discarded and wells were gently washed with PBS twice to remove any non-adherent cells. About 100 µl of fresh TSB broth containing one of, MIC, 2 × MIC and ½ MIC concentrations of each of tobacco tar were added to each well. Methanol was used as a control. The plates were covered to prevent evaporation and incubated at 25°C for 24 h. Broth containing the non-adherent cells were discarded through two rounds of washing with 200 µl sterile PBS buffer. Adherent cells to the plastic surfaces were quantified using Crystal violet assay. Experiment was performed in triplicate.

Effect of tobacco tar on the antimicrobial susceptibility of the tested strains

Cell suspensions (in sterile saline) of *S. aureus* strains that were grown on TSB with methanol (as a control), tobacoco tar and without tobacco tar for 24 h were tested for their susceptibility to some antibiotics (Penicillin, clindamycin, ceftriaxone, ciprofloxacin, erythromycin, amoxicillin/clavulanic acid, tetracycline, cefoperazone, Imipenem) using disc diffusion method (CLSI, 2011).

Cell suspensions of *C. albicans* strains were grown on TSB with methanol (as a control), Tobacoco tar and without tobacco tar for 24 h were tested for their antifungal susceptibility as follow: Using a sterile cotton swab, each isolate was plated onto a dried surface of a sterile Mueller-Hinton (Lab M, India) containing glucose and methylene blue agar plate, respectively. Antimicrobial disks of 10 µg of fluconazole, clotrimazole, itraconazole, amphotercin B and nystatin were dispensed onto the surface of the inoculated agar plate. Each disk was pressed down to ensure its complete contact with the agar surface. The plates were incubated at 37ºC and examined after 24 h of incubation. The zones of inhibition were measured in millimeter and the results were interpreted using validated CLSI interpretive values for *in vitro* susceptibility testing of antifungal agents (Kent, 1991).

Salt aggregation test for detection of cell surface hydrophobicity (CSH)

The cell suspensions of *S. aureus* and *C. albicans* (50 µl) were mixed with a series of dilutions of ammonium sulfate (50 µl) ranging from 0.2 to 2.0 mol/L. The lowest concentration of ammonium sulfate at which bacterial aggregation was visible was determined (using inverted microscope-binocular (0.32) at 20X magnification (Lecia, Germany). The clumps formed were scored positive on 1 to 4 scales (1+ to 4+). Strain was considered hydrophobic, if it aggregated in ammonium sulfate concentrations of ≤1.4 mol/L (Lindahl et al., 1981).

To detect effect of tobacco on the hydrophobicity of the tested microorganism, cells were grown on trypticase soy agar containing 0.04 g/ml of tobacco tar for 24 h and plates containing methanol as control; thereafter the previous test was repeated to determine the change of hydrophobicity.

Ethidium bromide accumulation assay

The effect of levofloxacin and ketoconazole on cell membrane permeability of *S. aureus* ATCC 29213 and *C. albicans* ATCC 10231 cells were evaluated by the method as described by Cox et al. (2000). The microbial cells were grown overnight in 100 ml of MHB at 37°C, washed and resuspended in 50 mmol/l sodium phosphate buffer, pH 7.1. The turbidity of the suspension was adjusted to 0.7 OD_{610} (1×10^9 CFU/ml). One milliliter volume of this suspension was added to flask containing 19 ml buffer and 0.04 gm/ml tobacco tar. Following 60 and 120 min incubation at room temperature, 200 µl were collected into Eppendorfs tubes containing 3.8 ml phosphate buffer. These tubes were stored on ice and 20 µl of ethidium bromide (Sigma) dissolved in milliQ water, was added to a final concentration of 10 µg/ml. Then, the fluorescence was measured using A Perkin Elmer LS 45 luminescence spectrometer (United Kingdom) equipped with 150 watt xenon arc lamp (1 cm quartz cell was used for spectrofluorimetric measurements and Slit width for both monochromators were set at 10 nm) (López-Amorós et al., 1995; Hilliard et al., 1999). The spectrometer is connected to a PC computer loaded with the FL WINLABtm software

Germ tube formation

C. albicans ATCC 10231 strain was cultivated on Sabouraud's dextrose agar for one day. Cells were suspended in sterile saline and adjusted to be equivalent to 0.5 McFarland turbidity. 400 µl of human serum was added to 1 ml of cell suspensions of *C. albicans* ATCC 10231. Tobacco tar was added to the suspension at MIC. In addition, saline was added to the control tube. The cell suspensions were incubated with gentle shaking at 37°C for 2 h and were examined for the presence of germ tubes by using a light microscope. Images for the morphological changes of the tested cells were taken by a DMRXA microscope (Leica, Germany) (Liu et al., 1994).

Real-time reverse transcription PCR (RT-PCR) analysis of *C. albicans* and *S. aureus* adhesion-related genes

Quantitative real-time reverse transcription PCR (RT-PCR) was used to detect the effect of tobacco tar on the adhesion-related genes. *C. albicans* ATCC 10231 was grown in sterile TSB with and

Table 1. Primers used for RT-PCR experiments.

Primer		Sequence	Real Time PCR conditions cycling or Tm (°C)
icaA	Probe	TGGATGTTGGTTCCAGAAACATTGGGAG	95°C 5 min (Hot Start activation); 40 × (95°C 5 s, annealing/extension 60°C 10 s)
	Forward	TGAACCGCTTGCCATGTG	
	Reverse	CACGCGTTGCTTCCAAAGA	
ALS1	Forward	5'-CCTATCTGACTAAGACTGCACC-3'	57.69
	Reverse	5'-ACAGTTGGATTTGGCAGTGGA-3'	60.13
HWP1	Forward	5'-CTCCAGCCACTGAAACACCA-3'	60.18
	Reverse	5'-GGTGGAATGGAAGCTTCTGGA-3'	60.00
β-actin gene (ACT1)	Forward	5'-CGTTGTTCCAATTTACGCTGGT-3'	60.03
	Reverse	5'-TGTTCGAAATCCAAAGCAACG-3'	58.01

without Tobacco tar. After incubation for 24 h at 37°C, the supernatant was discarded and the wells were washed twice with PBS. Total RNA was extracted from *C. albicans* biofilms using FastPure™ RNA kit (TaKaRa Biotechnology Co. Ltd, Dalian, China), according to the manufacturer's manual. Then, a BioPhotometer spectrophotometer (Eppendorf, Germany) was used to detect the concentrations and purity of the extracted RNA. Equal amounts of RNA were subjected to cDNA synthesis using the PrimeScript RT reagent kit (TaKaRa Biotechnology Co. Ltd, Dalian, China). Real-time PCR primers (Table 1) were designed for the target genes *ALS1*, *HWP1* using Primer Express 3.0 software (Applied Biosystems, CA, USA). Using β-actin gene (*ACT1*) as an endogenous reference. Real-time RT-PCR was performed with a StepOnePlus™ real-time PCR system (Applied Biosystems, CA, USA), and SYBR® Premix Ex Taq™ II was used as a reagent specifically designed for intercalator-based real-time PCR using SYBR Green I. PCR reaction mixture was as follow: 10 µl SYBR® Premix Ex TaqTM II (2X), 2 µl first strand cDNA, 0.5 µl each primer, 0.4 µl ROX Reference Dye (50X) and dH_2O to the final volume of 20 µl. The program for amplification was 95°C for 30 s as an initial denaturation step, followed by 40 cycles of PCR consisting of 95°C for 5 s and 60°C for 30 s. Negative controls (water) were included in each run. A melting curve was analyzed to confirm the specificity of each primer. Expression of each investigated gene was normalized to the housekeeping *ACT1* gene and analyzed using comparative Ct method (ΔΔCt). Expression of *ALS1* and *HWP1*, genes from cells grown under tobacco tar and ketoconazole (1/2 MIC each) treatment was indicated. Each experimental condition was performed in duplicate and each experiment was repeated twice.

For studying the effect of tobacco tar on the expression of *icaA* gene, Taqman quantitative Real-time PCR for the tested gene (Table 1) was performed. Probes were labeled with the reporter dye 6-carboxyfluorescein (6'-FAM) at the 5'end and with the quencher dye 6-carboxy-tetramethylrodamine (TAMRA) at the 3'end. Thermal cycling conditions were as follows: 2 min at 50°C, 10 min at 95°C followed by 45 repeats of 15 s at 95°C, and 1 min at 60°C. During each annealing phase, data was collected. Six-fold dilution serials of the cDNA mixture (from 50 ng to 5 pg) were used as the standards. All standard curves showed correlation coefficients of greater than 0.99, indicating a precise log-linear relationship. Expression of *ICA1* gene cells grown under tobacco tar and levofloxacin (1/2 MIC) treatment was indicated. Each experimental condition was performed in duplicate and each experiment was repeated twice.

Statistical analysis

Data were described as mean ± SD. All statistical analyses were performed by statistical analysis computer software package SPSS 17.0 (SPSS Inc., IL, USA). Student's t-test or one-way ANOVA were used to compare the biofilm formation, planktonic growth, and the gene expression of *C. albicans* and *S. aureus* strains in the presence or absence of drugs. Results with a p-value less than 0.05 were considered statistically significant.

RESULTS AND DISSCUSSION

Starting from lightening cigarette to inhaling a puff of smoke, various processes, that is, burning, pyrolysis, pyrosynthesis, distillation, sublimation and condensation occur (Borgerding and Klus, 2005). Cigarettes are filter tipped. Tip ventilation makes the mainstream smoke to be diluted with a defined amount of air during a puff. There are some factors that determine the chemical composition of cigarette smoke which are tobacco blend, cigarette paper, type and efficiency of the filter, and the degree of tip ventilation. When cigarettes are smoked, a complex mixture is inhaled into the respiratory system in the form of aerosol consisting of solids and liquid droplets (the particulate (''tar'') phase) in a gaseous phase. The quantities of the components of that smoke mixture ranged from milligramme (water, carbon monoxide, carbon dioxide and nicotine) to picogramme levels (heterocyclic amines and heavy metals) (Borgerding and Klus, 2005). Deposition of smoke components in the respiratory tract depends on the particle size. As larger particles deposited in the upper and larger airways, but those which are smaller penetrate deeper into the alveolar spaces. Ineffective clearance of this particulate matter causes particle retention in lung tissues, resulting in a chronic, low-grade inflammation that may be important in the progression of chronic lung diseases associated with long-term smoking (Ling and van Eeden, 2009). Also, microorganisms have been documented in cigarettes. As rapid growth of diverse bacteria and fungi, and microbial toxins accumulation were noticed after curing tobacco.

LC/MS analysis of tobacco tar

The LC/MS analysis of tobacco tar revealed the presence

Table 2. Components of tobacco tar by LC/ MS analysis.

R_t (min)	Peak m/z	Molecular formula	Compounds	S/N
0.52	104.059	C_8H_8	Styrene	1
0.53	191.055	$C_{10}H_{13}N_3O$	N-Nitrosoanabasine	2
0.54	178.055	$C_{14}H_{10}$	Phenanthrene	3
2.48	252.150	$C_{20}H_{12}$	Benzo[k]fluoranthene	4
2.57	110.060	$C_6H_6O_2$	Catechol	5
3.63	108.151	C_7H_8O	*p*-Cresol	6
3.63	110.071	$C_6H_6O_2$	Hydroquinone	7
3.64	138.114	$C_8H_{10}O_2$	2, 3-Dimethylhydroquinone	8
3.64	162.128	$C_{10}H_{14}N_2$	Nicotine	9
3.82	110.127	$C_6H_6O_2$	Resorcinol	10
3.83	167.127	$C_{12}H_9N$	Carbazole	11
4.37	154.275	$C_{12}H_{10}$	Acenaphthene	12
4.85	108.207	C_6H_4O	Benzoquinone	13
5.03	228.226	$C_{18}H_{12}$	Benz[a]anthracene	14
5.80	202.293	$C_{16}H_{10}$	Fluoranthene	15
5.97	354.265	$C_{16}H_{18}O_9$	Chlorogenic acid	16
6.61	202.221	$C_{16}H_{10}$	Pyrene	17
7.19	189.281	$C_{10}H_{11}N_3O$	N′-Nitrosoanatabine	18
8.25	177.245	$C_9H_{11}N_3O$	N′-Nitrosonornicotine	19
8.80	92.986	C_7H_8	Toluene	20

of 20 compounds. The tar contains nicotine, tobacco specific nitrosamines as N′-nitrosoanatabine, N′-nitrosonornicotine and N-nitrosoanabasine, polycyclic aromatic hydrocarbons as phenanthrene, benzo[k]fluoranthene, acenaphthene, benz[a]anthracene, fluoranthene and pyrene, in addition to other compounds as shown in Table 2 and Figures 1 to 5.

Determination of MIC of tobacco tar, levofloxacin and ketoconazole against *S. aureus* and *C. albicans* strains

Tobacco tar showed MICs against different strains of *S. aureus* higher than levofloxacin and double MICs against different strains *C. albicans* comparable with ketoconazole (Table 3), indicating that all tested *Candida* species were more sensitive to tobacco tar than bacteria.

Effect of tobacco tar on the microbial adherence

Tobacco tar showed a decrease in the adherence of the tested microorganisms occurred at concentrations above MIC. This is due to the growth inhibitory effect of the tobacco tar, while at low concentrations (sub MIC), the amount of adhered bacteria and fungi increased (Tables 4 and 5). The effect of cigarette smoke promoting cell adhesion in a dose dependent manner was previously reported by Baboni et al. (2009). The adhesion can be promoted by CSC compounds at certain concentration, but inhibited when these compounds are high explaining the decrease of *C. albicans* adhesion/biofilm formation at 40 and 50% of CSC.

Effect of tobacco tar on the antimicrobial susceptibility to some antimicrobials and the permeability of the tested organisms to ethidium bromide in the presence or absence of the standard antimicrobials

S. aureus exposed to tobacco tar expressed marked decrease in the susceptibility to penicillin, tetracycline and amoxicillin/clavulanic antibiotics (Table 6). While *C. albicans* exposed to tobacco tar showed marked decrease in susceptibility of most of the tested antifungals except for clotrimazole and ketoconazole (Table 7).

By studying the effect of tobacco tar on the permeability of the tested organisms to ethidium bromide in the presence of antimicrobials, it was found that the permeability to ethidium bromide was not affected after one hour, but it decreased after 2 h for both *S. aureus* (17.8 to 20%) and *C. albicans* (13.3 to 16.3%).

Salt aggregation test for detection of cell surface hydrophobicity (CSH)

Tobacco tar showed a marked increase of the hydrophobicity of the tested microorganisms by 2.5 to 7

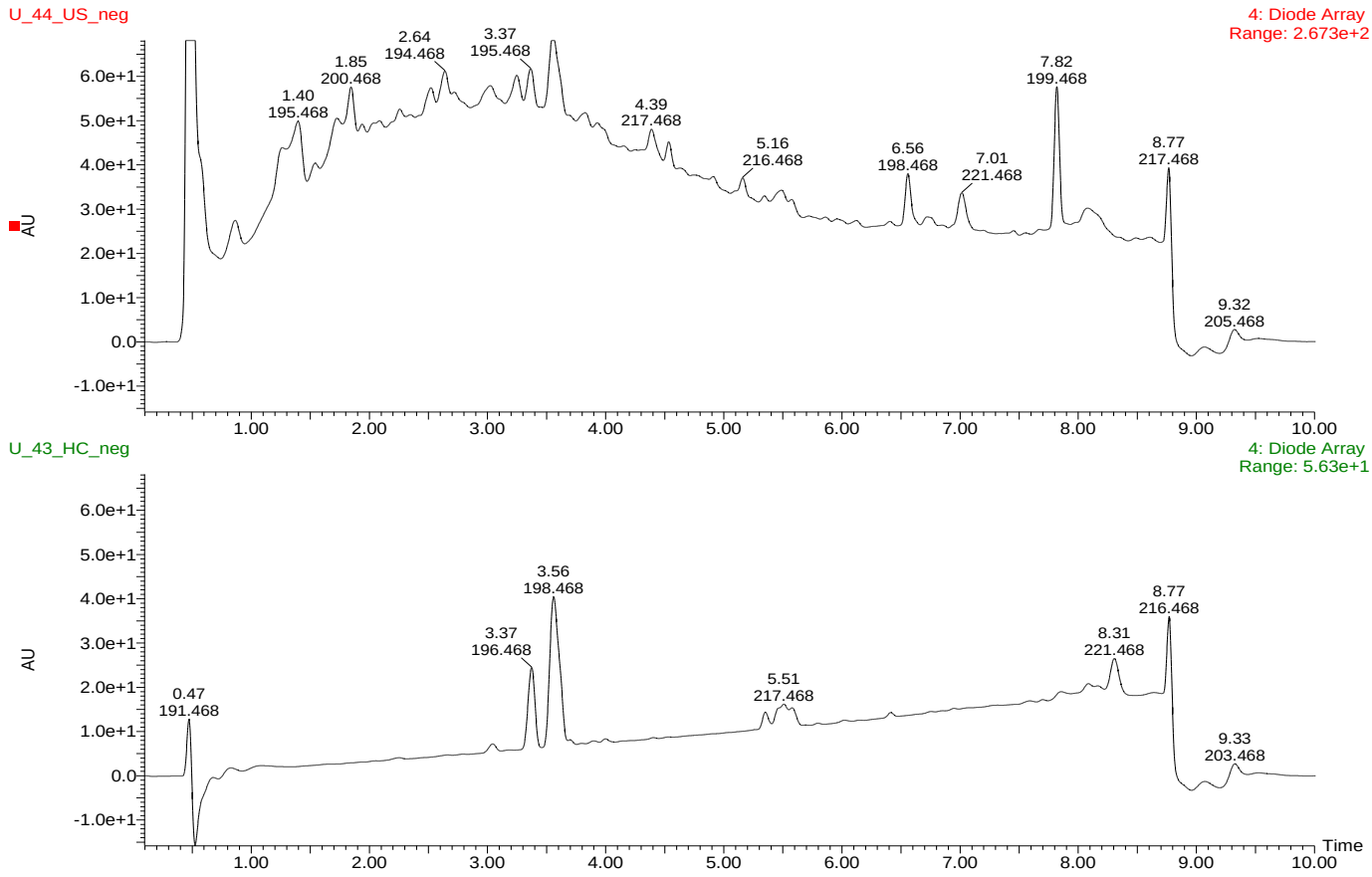

Figure 1. PDA chromatogram.

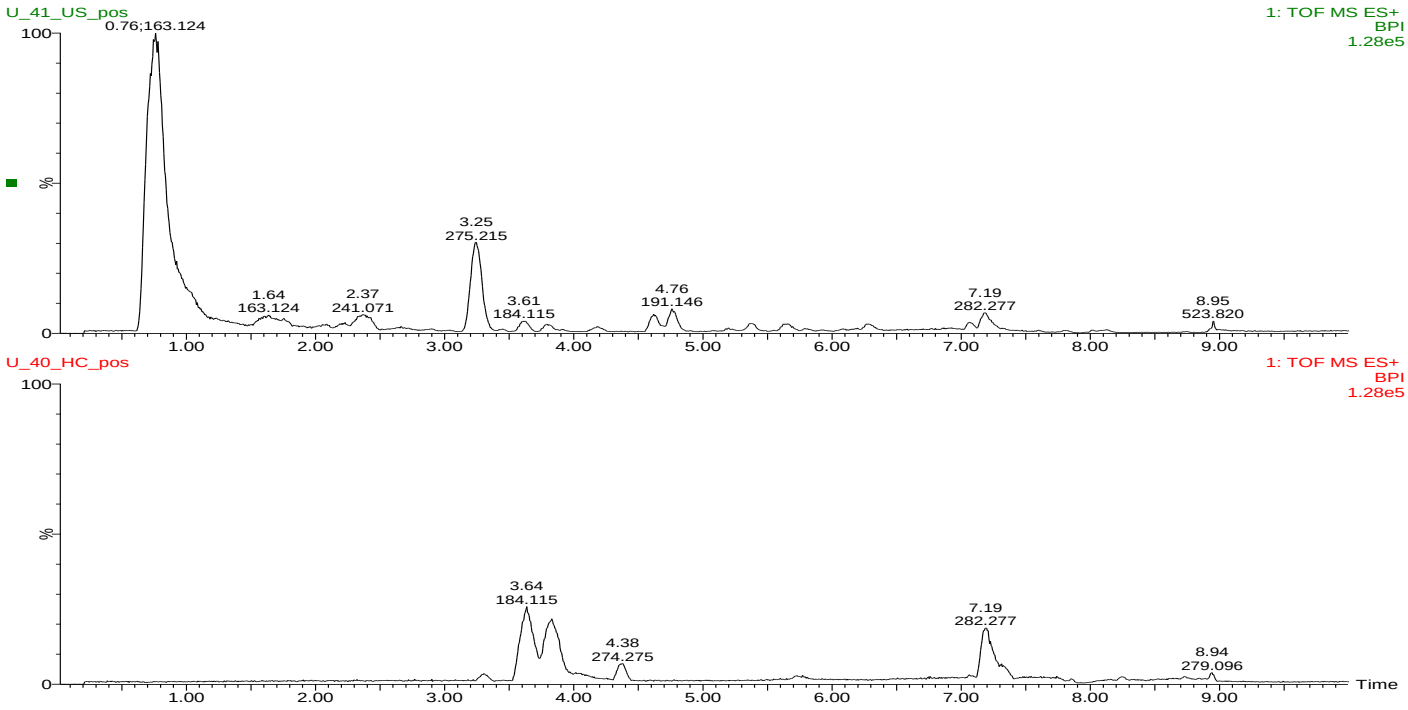

Figure 2. Positive ESI.

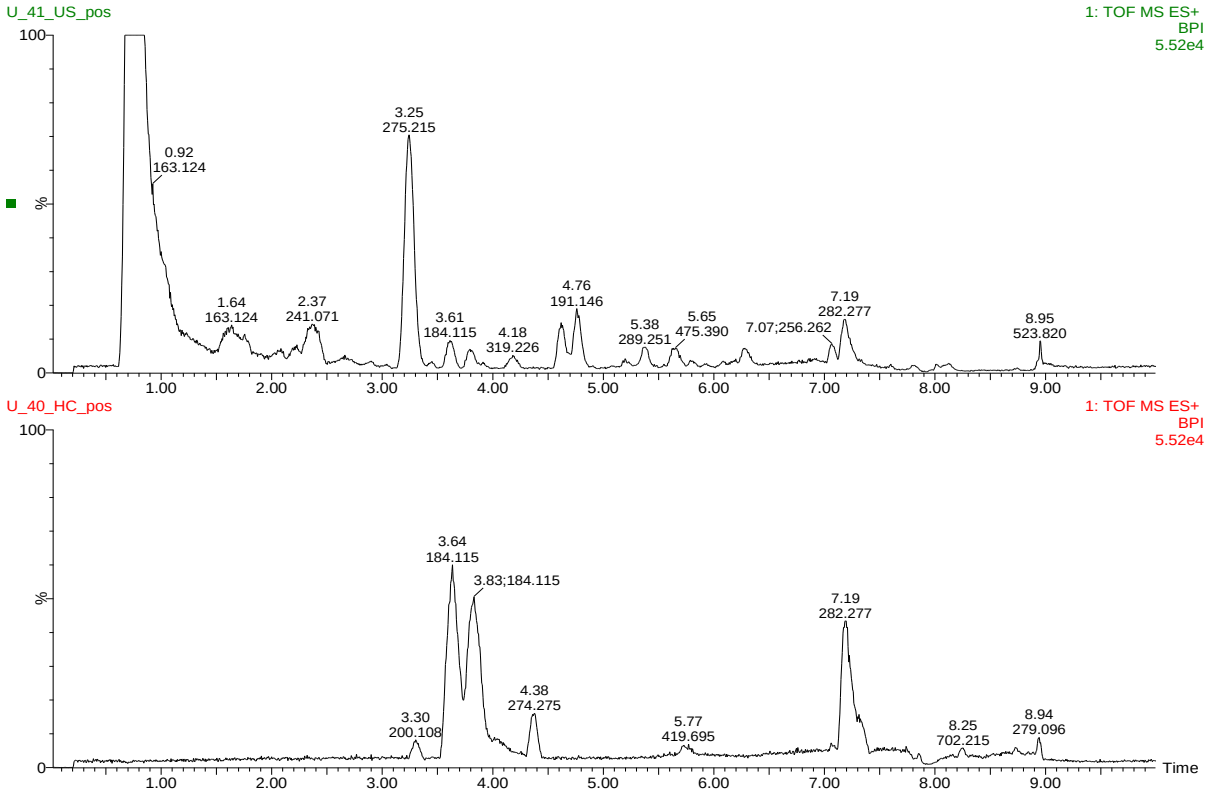

Figure 3. Positive ESI, zoomed in intensity (5.5E4 instead of 1.2E5).

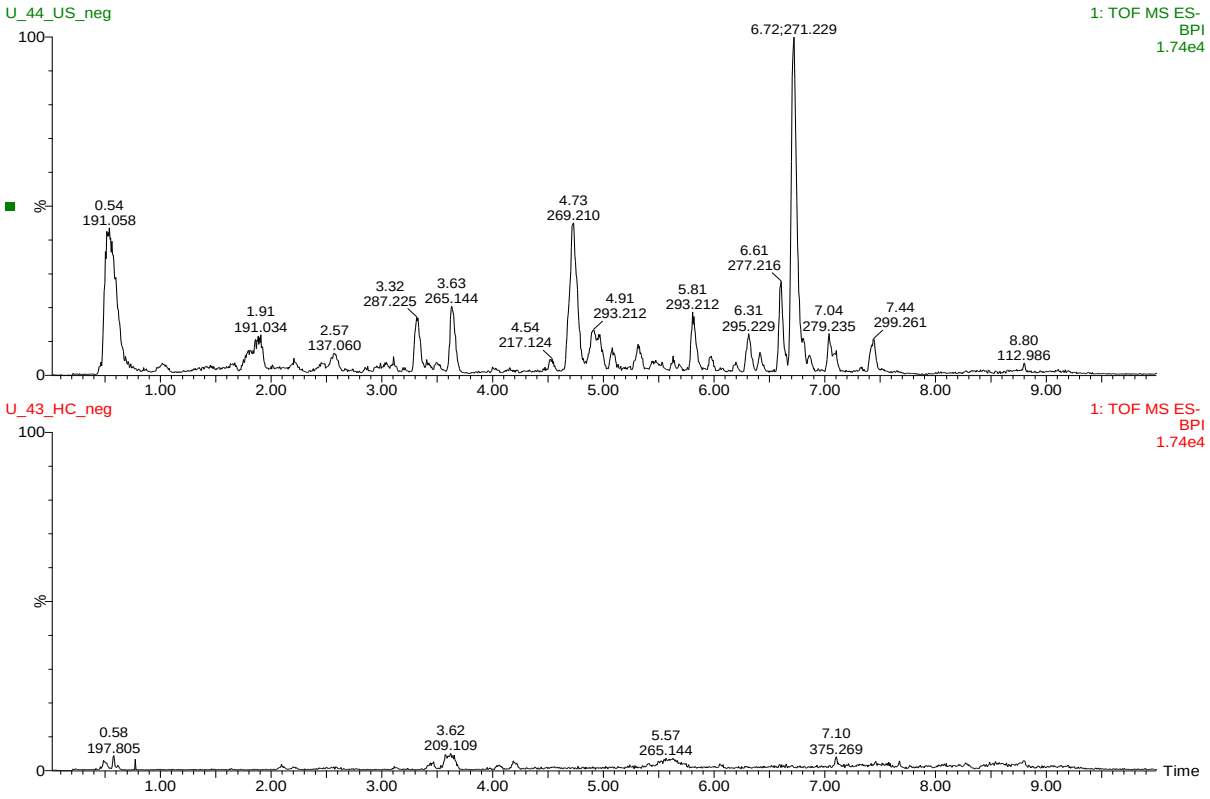

Figure 4. Negative ESI.

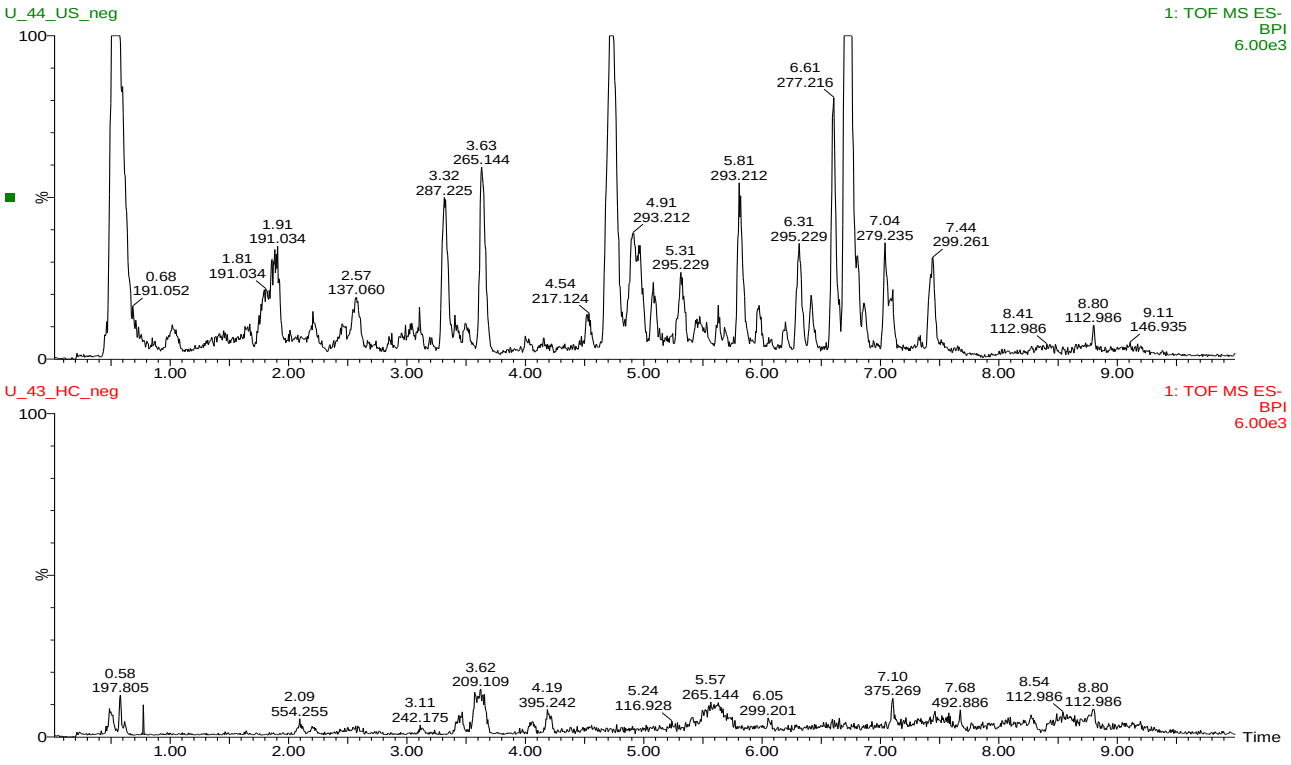

Figure 5. Negative ESI, zommed in the intensity to 6E3 instead of 1.7E4.

Table 3. MICs of tobacco tar, levofloxacin and ketoconazole against *S. aureus* and *C. albicans* strains.

Microorganism	Tobacco tar MIC (µg/ml)	Levofloxacin MIC (µg/ml)	Ketoconazole MIC (µg/ml)
S. aureus ATCC 29213	196.3	4.24	NT
S. aureus 1	160.7	18.6	NT
S. aureus 2	214.2	62	NT
C. albicans ATCC 10231	2.72	NT	1.25
C. albicans 1	4.015	NT	2.86
C. albicans 2	2.8	NT	2.2

MIC, Minimum inhibitory concentration; NT, not tested.

Table 4. Effect of tobacco tar on the *S. aurues* adherence.

Concentration of tobacco tar	*S. aureus* ATCC 29213	*S. aureus 1*	*S. aureus 2*
CTR	0.36±0.01	0.334±0.014	0.4±0.023
	0.161±0.056**	0.213±0.02	0.179±0.024**
2 X MIC	0.236±0.023*	0.177±0.031**	0.147±0.03**
	0.211±0.022*	0.186±0.03**	0.136±0.03**
MIC	0.269±0.012*	0.166±0.021**	0.20±0.051**
	0.45±0.0143**	0.193±0.05**	0.263±0.036*
1/2 MIC	0.626±0.024**	0.258±0.04*	0.467±0.022
	0.523±0.016**	0.546±0.02*	0.614±0.012**

Mean ± S.D, *significant at P < 0.05, **highly significant at p < 0.001.

Table 5. Effect of tobacco tar on the *C. albicans* adherence.

Concentration of tobacco tar	*C. albicans* ATCC 10231	*C. albicans 1*	*C. albicans 2*
CTR	0.3±0.034	0.324±0.04	0.422±0.013
	0.167±0.021**	0.188±0.021**	0.211±0.015**
2 X MIC	0.159±0.035**	0.272±0.012*	0.199±0.036**
	0.217±0.012	0.382±0.023	0.277±0.017*
MIC	0.265±0.013	0.323±0.045	0.376±0.011
	0.332±0.023	0.385±0.012	0.346±0.013
1/2 MIC	0.381±0.041	0.716±0.026**	0.399±0.03
	0.508±0.026**	0.462±0.014*	0.521±0.022**

Mean± S.D, *significant value P < 0.05, **highly significant p < 0.001.

Table 6. Antibacterial susceptibility of some antibiotics against *S. aureus* (S: not exposed to tobacco and S+T: exposed to tobacco).

Antibiotics	Growth inhibition zone (Mean± S.D)					
	***S. aurues** ATCC 29213*		***S. aureus 1***		***S. aureus 2***	
	S	**S+T**	**S**	**S+T**	**S**	**S+T**
Penicillin	9±0.01	5±0.023	12±0.06	10±0.02	18±0.02	13±0.02
Clindamycin	29±0.011	27±0.04	25±0.02	22±0.01	24±0.03	23±0.03
Ceftriaxone	28±0.03	26±0.02	24±0.05	24±0.01	27±0.01	25±0.02
Ciprofloxacin	22±0.02	22±0.023	29±0.01	28±0.03	26±0.2	26±0.01
Erythromycin	29±0.02	28±0.01	23±0.03	21±0.02	30±0.03	30±0.04
Amoxicillin/clavulanic	35±0.04	23±0.012	40±0.2	26±0.01	37±0.05	30±0.02
Tetracycline	17±0.01	10±0.014	20±0.01	11±0.04	15±0.02	8±0.03
Cefoperazone	15±0.05	13±0.04	16±0.03	16±0.034	18±0.04	16±0.01
Imipenem	45±0.04	45±0.26	50±0.1	49±0.02	43±0.02	43±0.04

Table 7. Antifungal susceptibility of some antifungals against *C. albicans* (C: not exposed to tobacco and C+T: exposed to tobacco).

Antifungal	Growth inhibition zone (Mean± S.D)					
	***C. albicans** ATCC 10231*		***C. albicans 1***		***C. albicans 2***	
	C	**C+T**	**C**	**C+T**	**C**	**C+T**
Amphotericin B	14±0.02	5±0.02	17±0.2	5±0.05	14±0.01	7±0.035
Nystatin	22±0.012	5±0.3	25±0.013	6±0.02	25±0.03	5±0.061
Fluconazole	12±0.2	5±0.03	14±0.02	5±0.013	15±0.034	5±0.032
Itraconazole	10±0.04	5±0.04	8±0.24	5±0.014	10±0.02	5±0.012
Clotrimazole	14±0.034	14±0.023	16±0.012	15±0.02	16±0.01	16±0.061
Ketoconazole	30±0.02	30±0.02	34±0.03	34±0.03	36±0.02	34±0.054

fold (Figure 6). AMPs (antimicrobial peptides) play important role as a part of the innate immunity in the eradication of bacteria. These peptides are produced by macrophages, neutrophils, and epithelial cells. Absence of AMPs makes macrophages to be less able to kill bacteria, and when bacterial growth is suppressed, bacteria are more resistant to AMP killing (Kristian et al., 2007). Thus, McEachern et al. (2015) found that slower-growing CSE-MRSA had low susceptibility to AMPs which may be the mechanism by which macrophages become less active against CSE MRSA. It was found that *S. aureus* can produce certain proteins that decrease hydrophobicity of its cell surface. These proteins are thought to be used by *S. aureus* to avoid AMP killing (Clarke et al., 2007). The effect of CSE on hydrophobicity was studied by McEachern et al. (2015) who found that CSE increased hydrophobicity in a dose-dependent manner, with fewer MRSA bacteria remaining in the aqueous layer. Hydrophobicity plays an important role in the interaction between microbial cells and epithelial cells

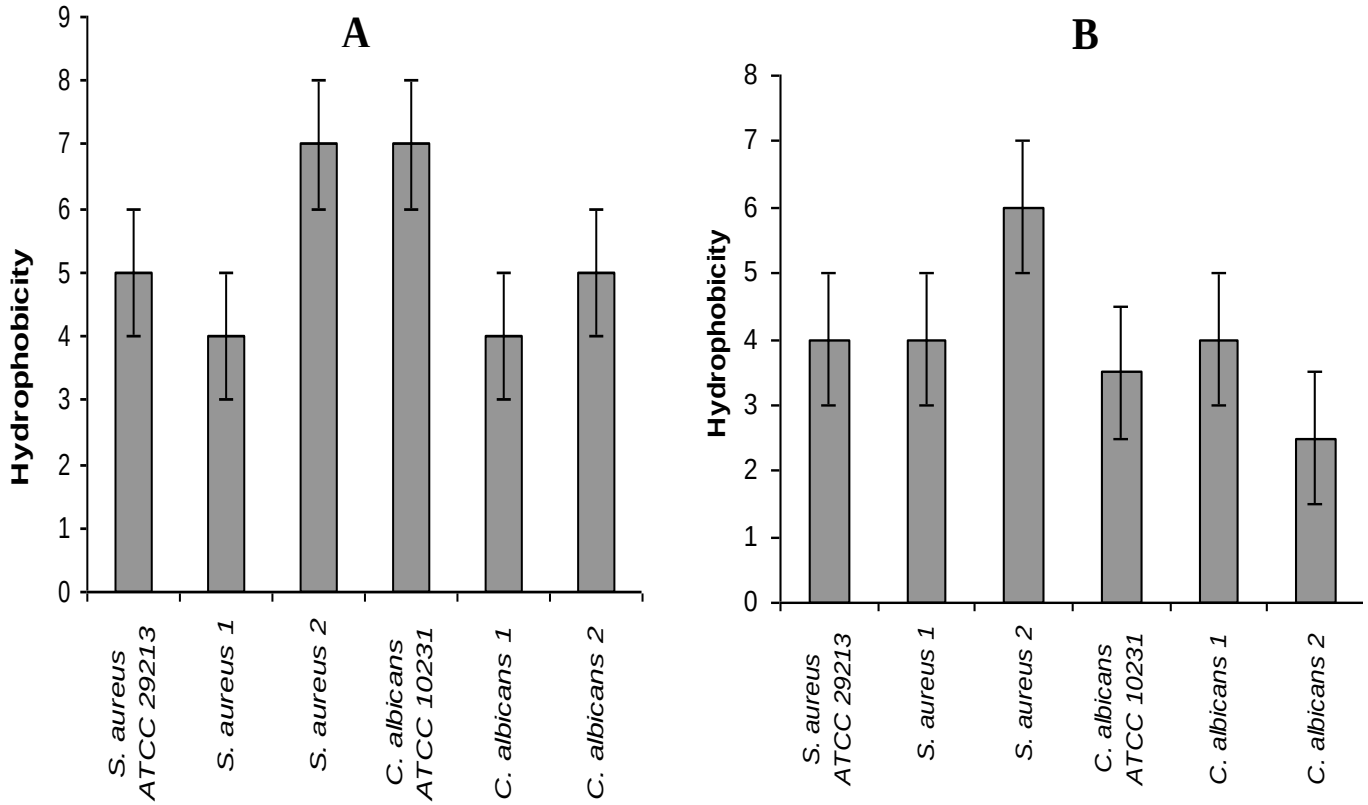

Figure 6. Effect of tobacco tar on the hydrophobicity of the tested microorganisms at ½ MIC (A) and ¼ MIC (B).

(Magnusson, 1982; Rosenberg and Kjelleberg, 1987; Dahlback et al., 1981). Increasing microbial hydrophobicity leads to the increase of microbial adherence to epithelial cells and their colonization which is considered the first step in developing invasive infections. Also, they found that cigarette made *S. aureus* with less negative surface charge that increased adherence and invasion of epithelial cells.

Effect of Tobacco tar on biofilm and Germ tube formation of C. albicans

C. albicans adhesion and growth are particularly necessary for biofilm formation (Inglis et al., 2013; Silva et al., 2011). Our study showed that CSC significantly increased *C. albicans* adhesion and growth and promoted biofilm formation. Interestingly, significant increase of biofilm formation was obtained at both tested concentrations, and that increase was found to be dependent on CSC concentration. These data are comparable to those of other studies showing increased microbial biofilm formation with cigarette smoke (Kulkarni et al., 2012; Mutepe et al., 2013; Bagaitkar et al., 2011). By showing the significant stimulatory effect on increasing *C. albicans* biofilm formation, cigarette smoke can thus be labeled as an infection-promoting agent.

By testing the effect of tobacco tar on germ tube formation, it was found that *C. albicans* showed germ tube formation even in the presence of tobacco while in the presence of ketoconazole no germ tube formed.

RT-PCR analysis of *C. albicans* and *S. aureus* adhesion-related genes

Tobacco tar increased or upregulated *ALS1* and *HWP1* genes that play an important role in adhesion, hyphae formation and biofilm formation. *S. aureus* biofilm formation requires the production of polymeric N-acetylglucosamine that is controlled by *icaABCD* operon. So, we tested the effect of tobacco on the expression of *icaA* gene, it was found that tobacco increase the expression of *icaA* gene that regulate biofilm formation (Table 8).

ALS1 is a member of the agglutinin-like sequence (ALS) gene family that encodes cell-wall glycoproteins. Most *ALS* proteins have adhesin functions (Sheppard et al., 2004; Zhao et al., 2005). Mutational analysis indicates that strains lacking all functional *ALS1* and *ALS3* alleles (als1Δ/als1Δ als3Δ/als3Δ) failed to produce any detectable adherent cells in biofilm models both *in vivo* and *in vitro* (Nobile et al., 2006), or in actual biofilm formation. *HWP1* is a well-characterized hypha specific

Table 8. Effect of the tested agents on the expression of adhesion-related genes.

Tested drugs	*C. albicans* (*ALS1*)		*C. albicans* (*HWP1*)		*S. aureus* (*icaA*)	
	Down-regulated (fold)	Up-regulated (fold)	Down-regulated (fold)	Up-regulated (fold)	Down-regulated (fold)	Up-regulated (fold)
Ketoconazole	1.197	-	3.89	-	NT	-
Ciprofloxacin	NT	-	NT	-	18	-
Tobacco Tar	2.065	-	-	3.18	-	1.2

NT, Not tested.

gene that can mediate *C. albicans* cell-cell interactions and improve biofilm formation (Sundstrom, 2002). Nobile et al. (2006) found that the expression of *HWP1* in *Saccharomyces cerevisiae* permits adherence to wild-type *C. albicans* but not an als1Δ/als1Δ als3Δ/als3Δ double mutant.

Promoting *C. albicans* adhesion, growth, and biofilm formation may operate through the modulated expression of certain *C. albicans* genes (Gutiérrez-Escribano et al., 2012; Rouabhia et al., 2012). CSC increased *HWP1* mRNA expression. *HWP1* is a downstream component of the cAMP-dependent PKA pathway and is positively regulated by EFG1 (Sharkey et al., 1999). The transcript level of *HWP1* increased with increasing CSC stimulation, which suggests that CSC did affect cAMP–EFG1 pathway activity, resulting in an increase of *C. albicans* adhesion and growth with biofilm formation. Further investigations are therefore warranted to gain greater insight into the interaction between cigarette smoke and *C. albicans* leading to infection.

Conclusion

Many of the components of tobacco tar have been identified, and compounds such as nicotine, tobacco specific nitrosamines and polycyclic aromatic hydrocarbons have been shown to be present in high concentrations. *C. albicans* was more susceptible to growth inhibition by tobacco tar than *S. aureus*. Furthermore, bacteria and fungi exposed to tobacco tar were shown to adhere more to epithelial cells at concentrations low MIC. Tobacco tar has been shown to promote microbial biofilm formation. Specifically, it interferes with *S. aureus* and *C. albicans* adhesion, resulting in biofilm formation, which suggests that cigarette smokers are more susceptible to life-threatening oral infections including candidiasis.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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