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Full Length Research Paper

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

Amany Hamouda Mahmoud[1], Mamdouh Nabil Samy[1], Amira Samir Wanas[1], Usama Ramdan Abdel-Mohsen[1], Rehab Mahmoud Abd El-Baky[2]* and Mohamed Salah Kamel[1]

[1]Department of Pharmacognosy, Faculty of Pharmacy, Minia University, Minia 61519, Egypt.
[2]Department of Microbiology and Immunology, Faculty of Pharmacy, Minia University, Minia 61519, Egypt.

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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

*Corresponding author. E-mail: dr_rehab010@yahoo.com, rehab.mahmoud@mu.edu.eg.

(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

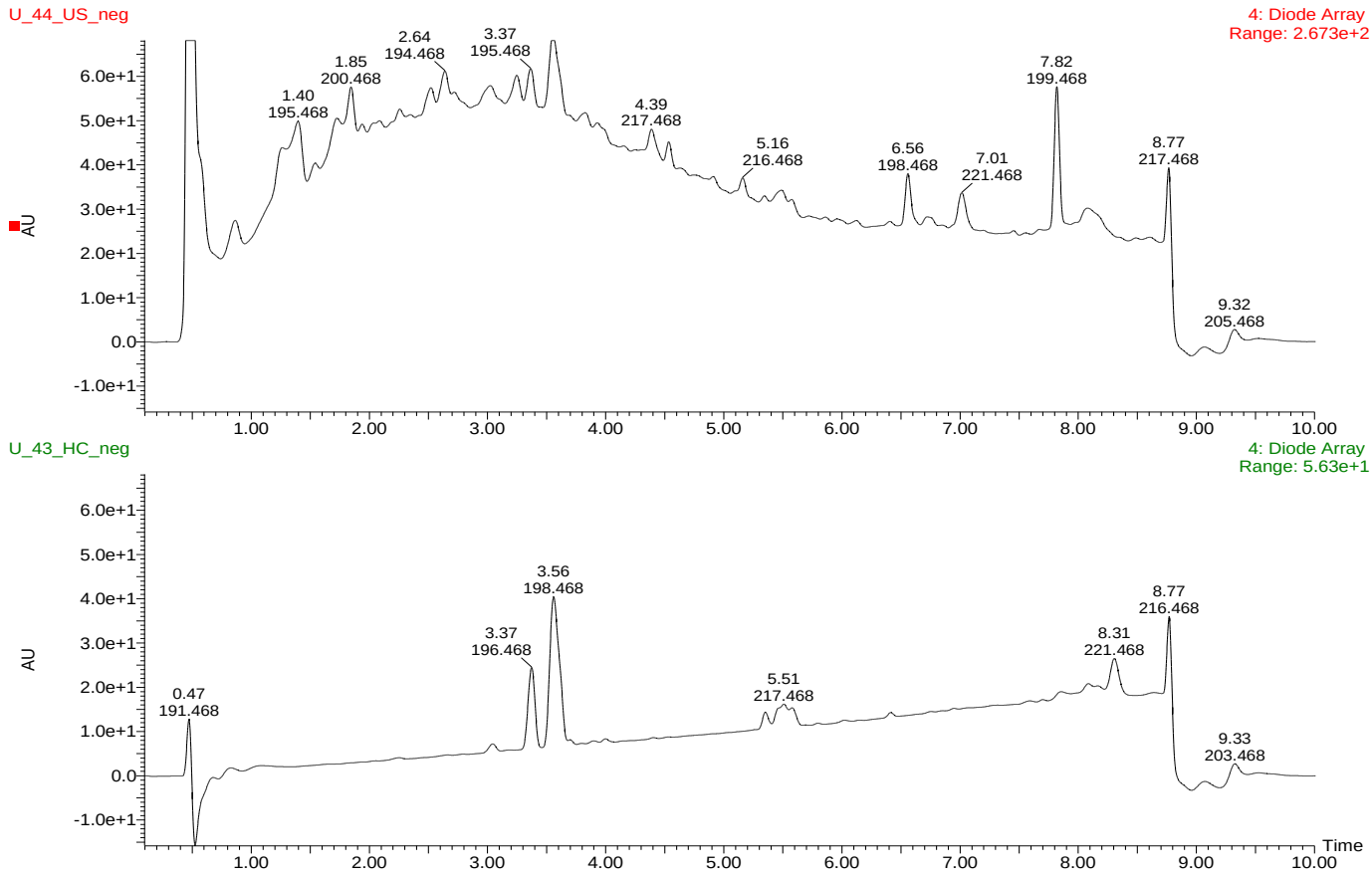

Figure 1. PDA chromatogram.

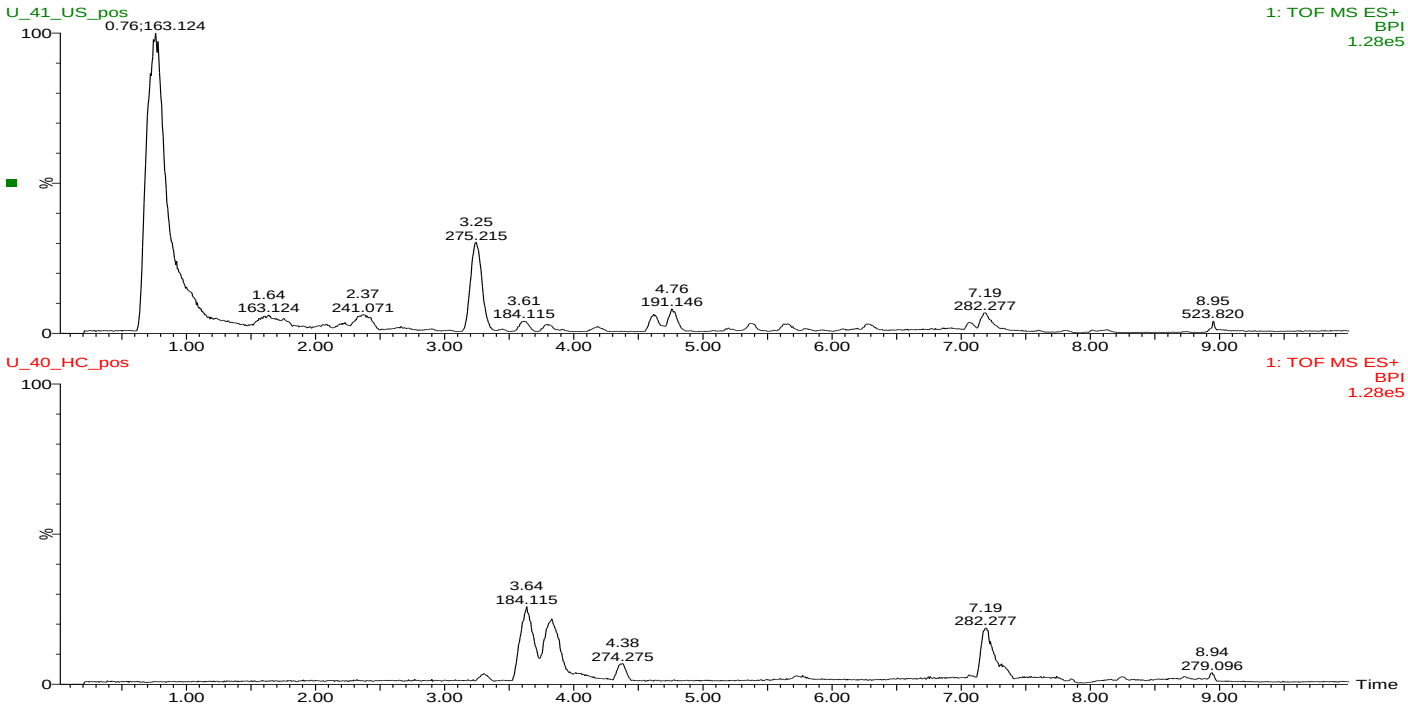

Figure 2. Positive ESI.

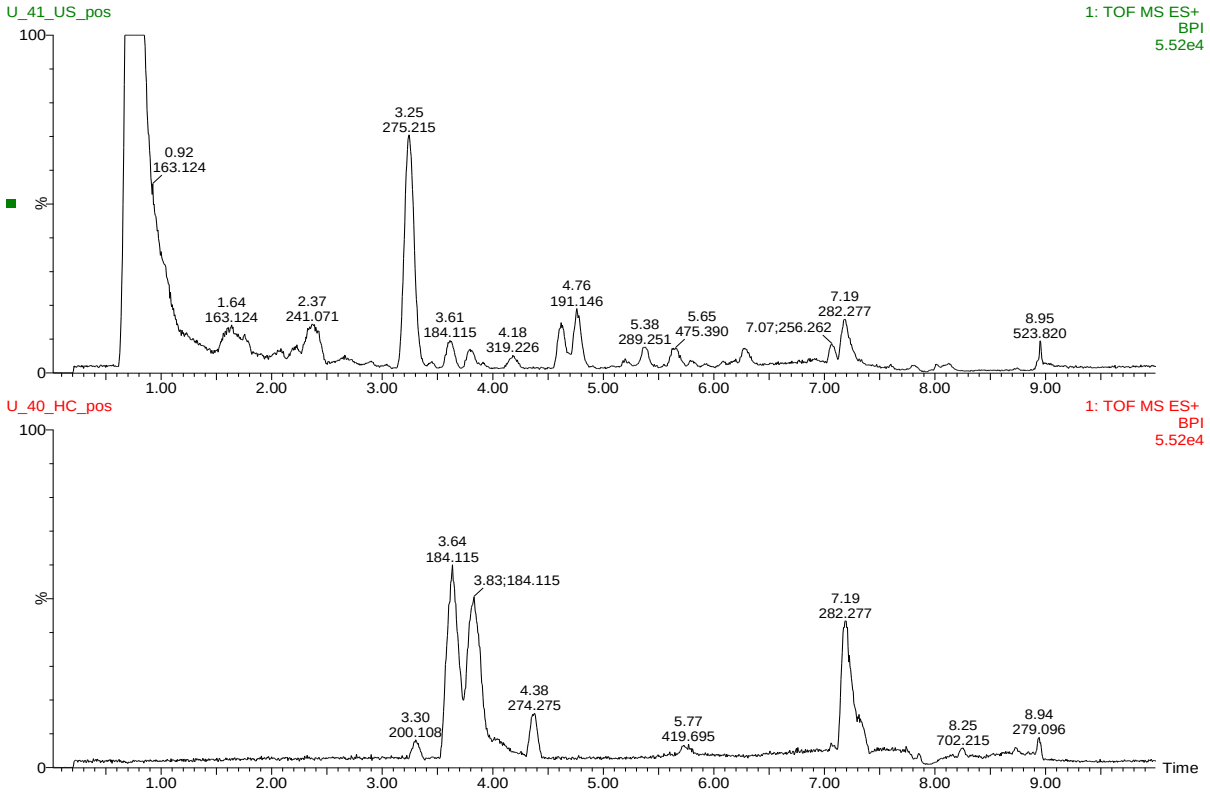

Figure 3. Positive ESI, zoomed in intensity (5.5E4 instead of 1.2E5).

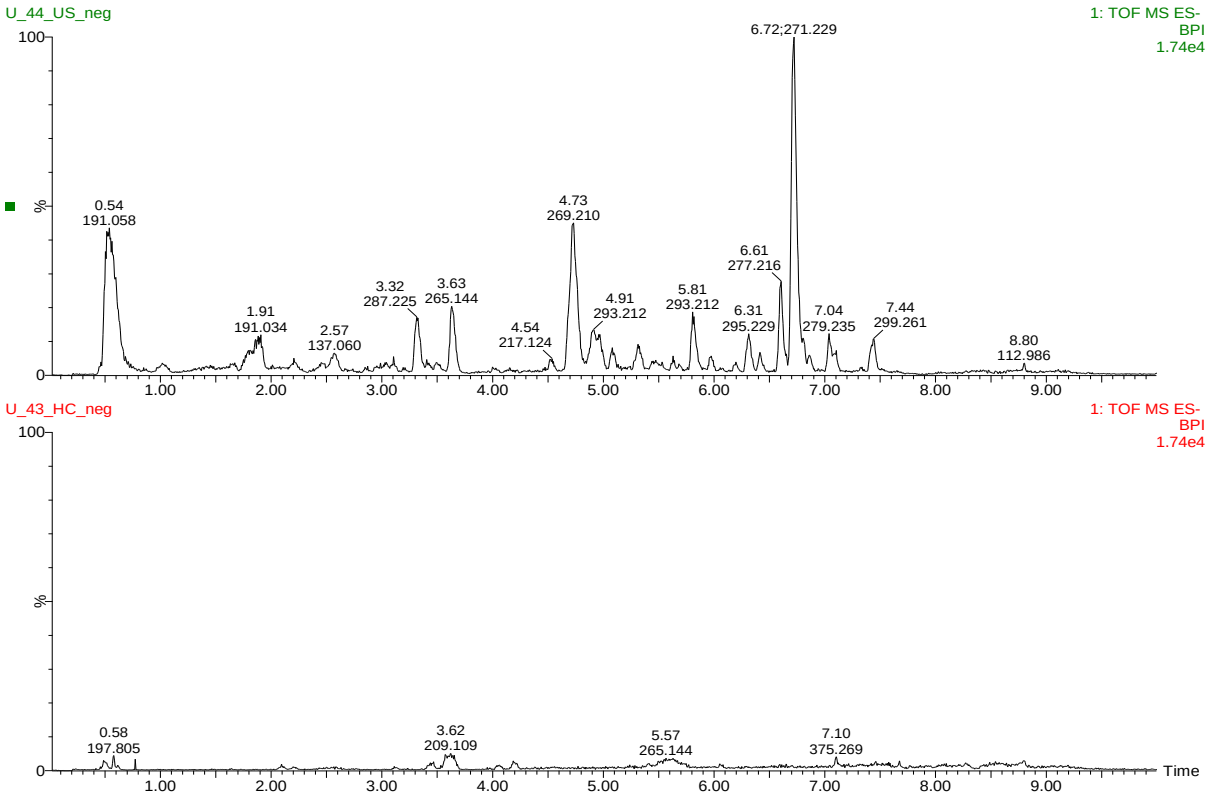

Figure 4. Negative ESI.

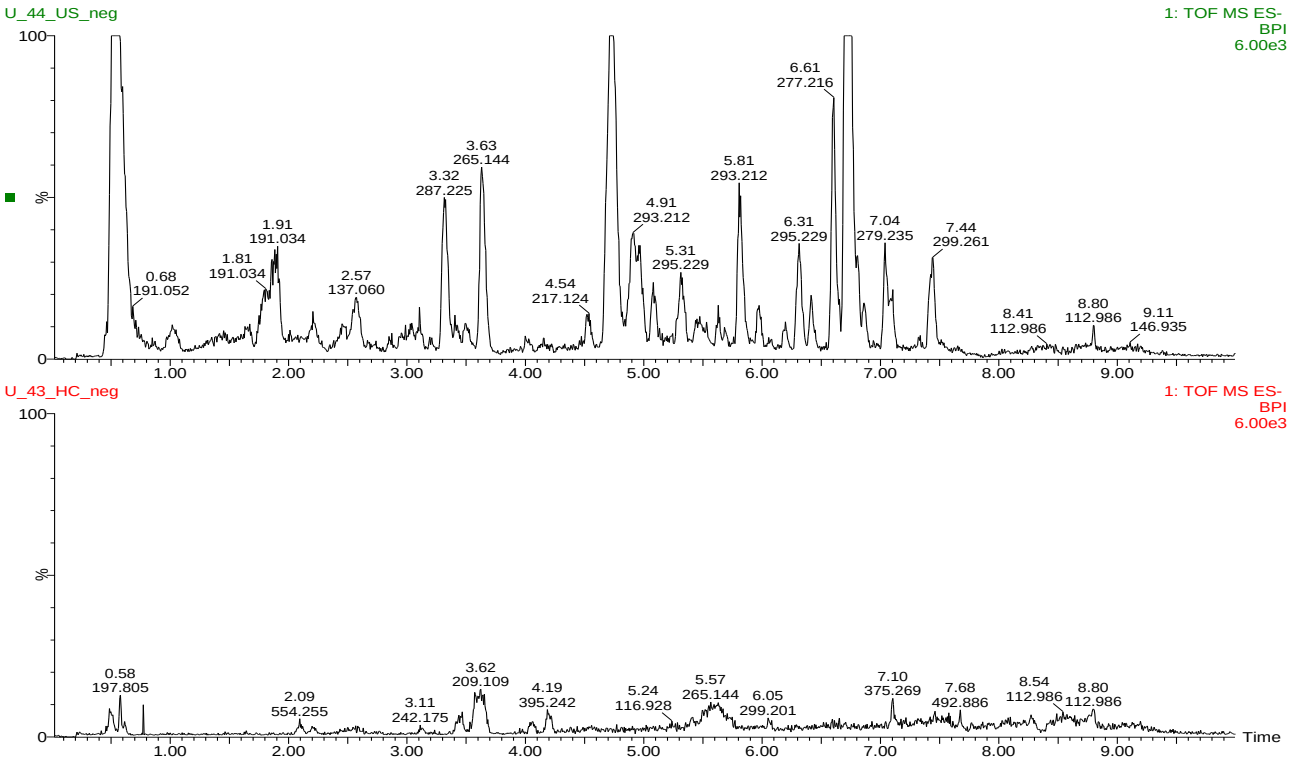

Figure 5. Negative ESI, zommed in the intensity to 6E3 instead of 1.7E4.

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

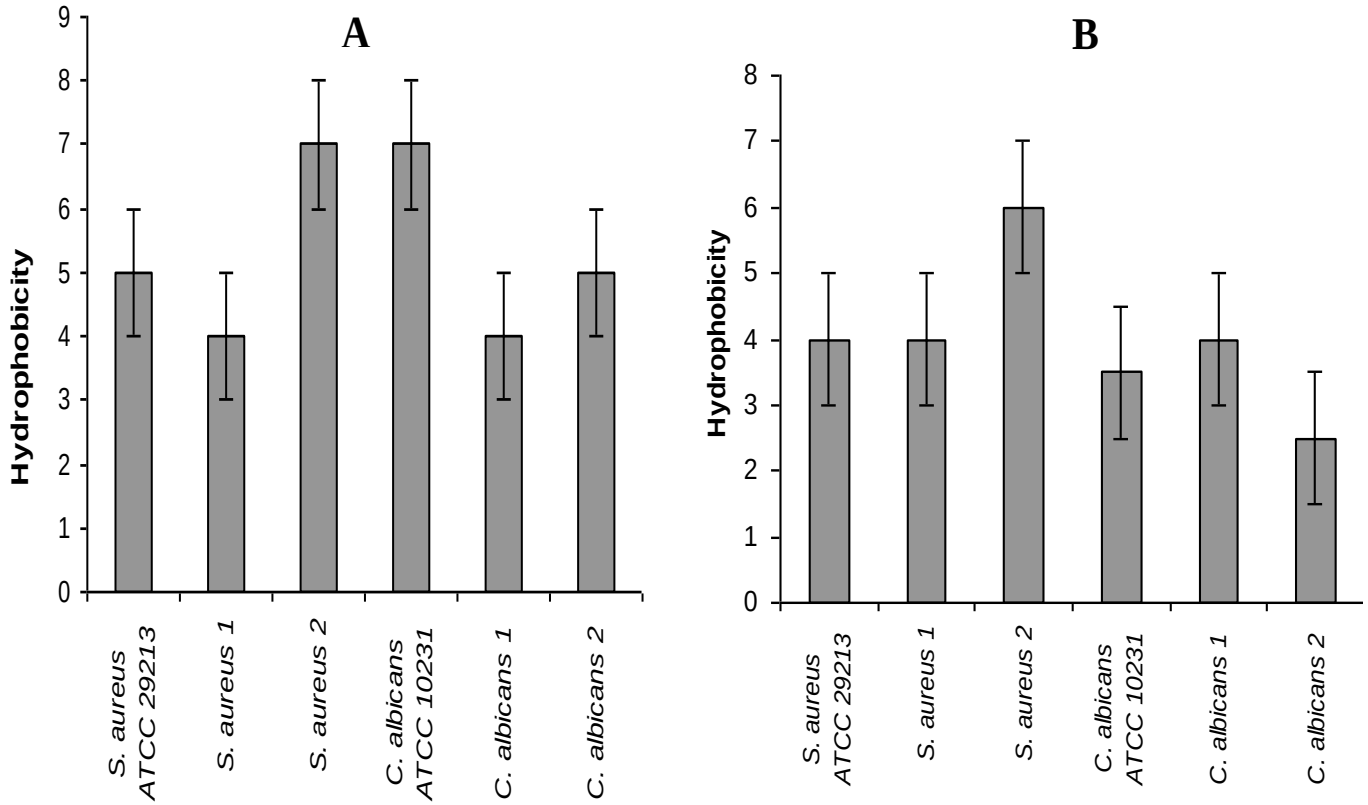

Figure 6. Effect of tobacco tar on the hydrophobicity of the tested microorganisms at ½ MIC (A) and ¼ MIC (B).

(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.

REFERENCES

Baboni FB, Barp D, Izidoro AC, Samaranayake LP, Rosa EA (2009). Enhancement of *Candida albicans* virulence after exposition to cigarette mainstream smoke. Mycopathologia 168:227-235.

Bagaitkar J, Daep CA, Patel CK, Renaud DE, Demuth DR, Scott DA, (2011). Tobacco smoke augments *Porphyromonas gingivalis-Streptococcus gordonii* biofilm formation. PLoS One 6:e27386.

Barnes PJ(2004). Mediators of chronic obstructive pulmonary disease. Pharmacol. Rev. 56: 515-548.

Borgerding M, Klus H (2005). Analysis of complex mixtures – cigarette smoke. Exp. Toxicol. Pathol. 57:43-73.

Boucher H, Miller LG, Razonable RR (2010). Serious infections caused by methicillin-resistant *Staphylococcus aureus.* Clin. Infect. Dis. 51(Suppl 2):S183-S197.

Centers for Disease Control and Prevention (2011). Current cigarette smoking prevalence among working adults-United States, 2004-2010 MMWR Morb. Mortal Wkly. Rep. 60:1305-1309.

Christensen GD, Simpson WA, Younger JA, Baddour LM, Barrett FF, Melton DM, Beachey EH, (1985). Adherence of coagulase negative *Staphylococci* to plastic tissue cultures: a quantitative model for the adherence of *Staphylococci* to medical devices. J. Clin. Microbiol. 22:996-1006.

Church DF, Pryor WA, (1985). Free-radical chemistry of cigarette smoke and its toxicological implications. Environ. Health Perspect. 64:111-126.

Clarke SR, Mohamed R, Bian L, Routh AF, Kokai-Kun JF, Mond JJ, Tarkowski A, Foster SJ (2007). The *Staphylococcus aureus* surface protein IsdA mediates resistance to innate defenses of human skin. Cell Host Microbe 1:199-212.

CLSI-Clinical and Laboratory Standards Institutes (2011). Performance standards for antimicrobial susceptibility testing Twenty first information supplement M100-S21Wayne, PA: CLIS:2011

Cosı´o MG, Saetta M, Agustı´ A (2009). Immunologic aspects of chronic obstructive pulmonary disease. N. Engl. J. Med. 360:2445-2454

Cox SD, Mann CM, Markham JL, Bell HC, Gustafson JE, Warmington JR, Wyllie SG (2000). The mode of antimicrobial action of the essential oil of *Melaleuca alternifolia* (tree oil). J. Appl. Microbiol 88:170-175.

Dahlback B, Hermansson M, Kjelleberg S, Norkrans B (1981). The hydrophobicity of bacteria-an important factor in their initial adhesion at the air-water interface. Arch. Microbiol. 128:267-270.

Delahaye C, Rainford L, Nicholoson A, Mitchell S, Lindo J, Ahmad M (2009). Antibacterial and antifungal analysis of crude extracts from the leaves of *Callistemon viminalis.* J. Med. Biol. Sci. 3:1-7.

Ebbert JO, Croghan IT, Schroeder DR, Murawski J, Hurt RD (2007). Association between respiratory tract diseases and secondhand smoke exposure among never smoking flight attendants: a cross-sectional survey. Environ. Health 6:28.

Feng Y, Kong Y, Barnes PF, Huang FF, Klucar P, Wang X, Samten, B, Sengupta M, Machona B, Donis R, Tvinnereim AR, Shams H (2011). Exposure to cigarette smoke inhibits the pulmonary T-cell response to influenza virus and *Mycobacterium tuberculosis.* Infect. Immun. 79:229-237.

Fidel PL Jr (2011). *Candida*-host interactions in HIV disease: implications for oropharyngeal candidiasis. Adv. Dent. Res. 1:45-49.

Gutiérrez-Escribano P, Zeidler U, Suárez MB, Bachellier-Bassi S, Clemente-Blanco A, Bonhomme J, de Aldana CRV, d'Enfert C, Correa-Bordes J (2012). The NDR/LATS kinase Cbk1 controls the activity of the transcriptional regulator Bcr1 during biofilm formation in

Candida albicans. PLoS Pathog. 8:e1002683.
Heilmann C (2011). Adhesion mechanisms of *Staphylococci.* Adv. Exp. Med. Biol. 715:105-123.
Hilliard JJ, Goldschmidt RM, Licata L, Baum EZ, Bush K (1999). Multiple mechanisms of action for inhibitors of histidine protein kinases from bacterial two-component systems Antimicrob. Agents Chemother. 43:1693-1699.
Hogg JC, Chu F, Utokaparch S, Woods R, Elliott WM, Buzatu L, Cherniack RM, Rogers RM, Sciurba FC, Coxson HO, Paré PD (2004). The nature of small-airway obstruction in chronic obstructive pulmonary disease. N. Engl. J. Med. 350:2645-2653.
Huvenne W, Lanckacker EA, Krysko O, Bracke KR, Demoor T, Hellings PW, Brusselle GG, Joos GF, Bachert C, Maes T (2011). Exacerbation of cigarette smoke-induced pulmonary inflammation by *Staphylococcus aureus* enterotoxin B in mice. Respir. Res. 12:69.
Inglis DO, Skrzypek MS, Arnaud MB, Binkley J, Shah P, Wymore F, Sherlock G (2013). Improved gene ontology annotation for biofilm formation, filamentous growth, and phenotypic switching in *Candida albicans.* Eukaryot. Cell 12:101-108.
Kent HL (1991). Epidemiology of vaginitis. Am. J. Obstet. Gynecol. 165:1168-1176.
Kristian SA, Timmer AM, Liu GY, Lauth X, Sal-Man N, Rosenfeld Y, Shai Y, Gallo RL, Nizet V (2007). Impairment of innate immune killing mechanisms by bacteriostatic antibiotics. FASEB J. 21:1107-1116.
Kulkarni R, Antala S, Wang A, Amaral FE, Rampersaud R, Larussa SJ, Planet PJ, Ratner AJ, (2012). Cigarette smoke increases *staphylococcus aureus* biofilm formation via oxidative stress. Infect. Immun. 80:3804-3811.
Kulkarni R, Rampersaud R, Aguilar JL, Randis TM, Kreindler JL, Ratner, AJ, (2010). Cigarette smoke inhibits airway epithelial cell innate immune responses to bacteria. Infect. Immun. 78:2146-2152.
Lindahl M, Faris A, Wadström T, Hjertén S (1981). A new test based on 'salting out' to measure relative surface hydrophobicity of bacterial cells. Biochim. Biophys. Acta 677:471-476.
Ling SH, van Eeden SF (2009). Particulate matter air pollution exposure: role in the development and exacerbation of chronic obstructive pulmonary disease. Int. J. Chron. Obstruct. Pulmon. Dis. 4:233-243.
Liu H, Kohler J, Fink GR (1994). Suppression of hyphal formation in *Candida albicans* by mutation of a STE12 homolog. Science 266:1723-1726.
López-Amorós R, Comas J, Vives-Rego J (1995). Flow cytometric assessment of *Escherichia coli* and *Salmonella typhimurium* starvation-survival in seawater using rhodamine 123, propidium iodide, and oxonol. Appl. Environ. Microbiol. 61:2521-2526.
Lowy FD (1998). *Staphylococcus aureus* infections. N. Engl. J. Med. 339:520-532.
Magnusson KE (1982). Hydrophobic interaction–a mechanism of bacterial binding. Scand. J. Infect. Dis. Suppl. 33:32-36.
McEachern EK, Hwang JH, Sladewski KM, Nicatia S, Dewitz C, Mathew DP, Nizet V, Crotty Alexander LE (2015). Analysis of the effects of cigarette smoke on staphylococcal virulence phenotypes. Infect. Immun. 83:2443-2452.
Mizgerd JP (2008). Acute lower respiratory tract infection. N. Engl. J. Med. 358:716-727.
Morschhauser J (2010). Regulation of multidrug resistance in pathogenic fungi. Fungal Genet. Biol. 47:94-106.
Murphy TF, Bakaletz LO, Smeesters PR (2009). Microbial interactions in the respiratory tract. Pediatr. Infect. Dis. J. 28:S121-S126.
Mutepe ND, Cockeran R, Steel HC, Theron AJ, Mitchell TJ, Feldman C, Anderson R (2013). Effects of cigarette smoke condensate on pneumococcal biofilm formation and pneumolysin. Eur. Respir. J. 41:392-395.
Nave R, Mueller H (2103). From inhaler to lung: clinical implications of the formulations of ciclesonide and other inhaled corticosteroids. Int. J. Gen. Med. 6:99-107.
Nobile CJ, Nett JE, Andes DR, Mitchell AP (2006). Function of *Candida albicans* adhesin Hwp1 in biofilm formation. Eukaryotic Cell 5(10):1604-1610.
Ogbulie JN, Ogueke CC, Okaoli IC Anyanwan BN, (2007). Antibacterial activities and toxicological potentials of crude ethanolic extracts of *Euphorbia hirta.* Afr. J. Biotechnol. 6:1544-1548.
O'Toole AG, Kolter R (1998). Initiation of biofilm formation in *Pseudomonas fluorescens* WCS365 proceeds via multiple, convergent signaling pathways: a genetic analysis. Mol. Microbiol. 28:449-461.
Pinel B, Cassou-Mounat T, Bensadoun RJ (2012). Oropharyngeal candidiasis and radiotherapy. Cancer Radiother. 3:222-229.
Prasad DS, Kabir Z, Dash AK, Das BC (2009). Smoking and cardiovascular health: a review of the epidemiology, pathogenesis, prevention and of tobacco. Indian J. Med. Sci. 63:520-533.
Pryor WA, Stone K, Zang LY, Bermudez E (1998). Fractionation of aqueous cigarette tar extracts: fractions that contain the tar radical cause DNA damage. Chem. Res. Toxicol. 11: 441-448.
Richardson M, Lass-Florl C (2008). Changing epidemiology of systemic fungal infections. Clin. Microbiol. Infect. 14:5-24.
Rosenberg M, Kjelleberg S (1987). Hydrophobic Interactions: Role in Bacterial Adhesion, In. Marshall, KC (ed), Advances in Microbiology Ecology, vol 9 Plenum, New York, NY.
Rouabhia M, Ross G, Page N, Chakir J (2002). Interleukin-18 and gamma interferon production by oral epithelial cells in response to exposure to *Candida albicans* or lipopolysaccharide stimulation. Infect. Immun. 70:7073-7080.
Rouabhia M, Schaller M, Corbucci C, Vecchiarelli A, Prill SK, Giasson L, Ernst JF (2005). Virulence of the fungal pathogen *Candida albicans* requires the five isoforms of protein mannosyl transferases. Infect. Immun. 73:4571-4580.
Rouabhia M, Semlali A, Chandra J, Mukherjee P, Chmielewski W, Ghannoum MA (2012). Disruption of the *ECM33* gene in *Candida albicans* prevents biofilm formation, engineered human oral mucosa tissue damage and gingival cell necrosis/apoptosis. Mediators Inflamm. 2012:398207
Saunus JM, Kazoullis A, Farah CS (2008). Cellular and molecular mechanisms of resistance to oral *Candida albicans* infections. Front. Biosci. 13:5345-5358.
Sethi S, Murphy TF (2001). Bacterial infection in chronic obstructive pulmonary disease in 2000: a state-of-the-art review. Clin. Microbiol. Rev. 14:336-363.
Sharkey LL, McNemar MD, Saporito-Irwin SM, Sypherd PS, Fonzi WA (1999). HWP1 functions in the morphological development of *Candida albicans* downstream of EFG1, TUP1, and RBF1. J. Bacteriol. 181:5273-5279.
Sheppard DC, Yeaman MR, Welch WH, Phan QT, Fu Y, Ibrahim AS, Filler SG, Zhang M, Waring AJ, Edwards JE Jr (2004). Functional and structural diversity in the Als protein family of *Candida albicans.* J. Biol. Chem. 279:30480-30489.
Shopsin B, Mathema B, Martinez J, Ha E, Campo ML, Fierman A, Krasinski K, Kornblum J, Alcabes P, Waddington M, Riehman M, Kreiswirth BN (2000). Prevalence of methicillin-resistant and methicillin susceptible *Staphylococcus aureus* in the community. J. Infect. Dis. 182:359-362.
Silva S, Negri M, Henriques M, Oliveira R, Williams DW, Azeredo J (2011). Adherence and biofilm formation of non-*Candida albicans Candida* species. Trends Microbiol. 19:241-247.
Soysa NS, Ellepola AN (2005). The impact of cigarette/tobacco smoking on oral candidosis: an overview. Oral Dis 5:268-273.
Stampfli MR, Anderson GP (2009). How cigarette smoke skews immune responses to promote infection, lung disease and cancer. Nat. Rev. Immunol. 9:377-384.
Sundstrom P (2002). Adhesion in *Candida* spp. Cell Microbiol. 4:461-469.
Warren CW, Jones NR, Peruga A, Chauvin J, Baptiste, JP, Costa de Silva, V, el Awa F, Tsouros A, Rahman K, Fishburn B, Bettcher DW, Asma S (2008). Global youth tobacco surveillance, 2000–2007. MMWR Surveill. Summ. 57:1-28.
Wertheim HF, Melles DC, Vos MC, van Leeuwen W, van Belkum A, Verbrugh HA, Nouwen JL (2005). The role of nasal carriage in *Staphylococcus aureus* infections. Lancet Infect. Dis. 5:751-762.
Yageta Y, Ishii Y, Morishima Y, Masuko H, Ano S, Yamadori T, Itoh, K, Takeuchi K, Yamamoto M, Hizawa N (2011). Role of Nrf2 in host defense against influenza virus in cigarette smoke-exposed mice. J. Virol. 85:4679-4690.
Yao H, Rahman I (2011). Current concepts on oxidative/carbonyl stress,

inflammation and epigenetics in pathogenesis of chronic obstructive pulmonary disease. Toxicol. Appl. Pharmacol. 254:72-85.
Zhao X, Oh SH, Yeater KM, Hoyer LL (2005). Analysis of the Candida albicans Als2p and Als4p adhesins suggests the potential for compensatory function within the Als family. Microbiology 151:1619-1630.

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In vitro cytotoxicity and biological activities of *Genipa americana* (Rubiaceae) ethanolic extracts

Paola Silva Campos Codignoto[1], Shirley Batista de Araújo[1], Natan Munhoz Bastos[1], Thais de Oliveira Fernandes[4], Tamiris Arielle Santana Barbosa[4], Carlos Eduardo Dias Igidio[2], Fernando Faustino[3], Maria Judite Bittencourt Fernandes[1] and Aline Oliveira da Conceição[2*]

[1]Centro de Pesquisa e Desenvolvimento de Sanidade Animal – Instituto Biológico, São Paulo/SP, Brazil.
[2]Departamento de Ciências Biológicas – Universidade Estadual de Santa Cruz, Ilhéus/BA, Brazil.
[3]Departamento de Ciências Exatas e Tecnológicas – Universidade Estadual de Santa Cruz, Ilhéus/BA, Brazil.
[4]Departamento de Ciências Agrárias e Ambientais – Universidade Estadual de Santa Cruz, Ilhéus/BA, Brazil.

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This study aimed to determine the *in vitro* toxicity of *Genipa americana* and its antiviral and anti-microbial activities. Due to lack of knowledge on *G. americana* validation as medicinal plant, biological studies were performed. For the cytotoxicity experiments, decreasing concentrations of ethanolic extracts of *G. americana* branches, fruits, and leaves were added to a confluent monolayer of Vero cells. After 72 h, it was determined, the first concentration of each extract in which any cellular change was observed (maximum non-cytotoxic concentration- MNCC). The MNCC of fruit and branches extracts was of 0.5 and 0.25 mg/mL, respectively. The leaves extract did not show cytotoxicity in the tested dilutions. The antiviral activities of *G. americana* extracts were tested on the suid (SuHV-1) and equine (EHV-1) alpha herpes viruses through reduction of their viral titers. The fruit extract presented a viral inhibition on EHV-1 of 86%, but it was not effective for SuHV-1. Instead, the leaves and branch extracts showed antiviral activity against SuHV-1, with a viral inhibition of 73 and 79%, respectively, but no inhibition on EHV-1. The antimicrobial action on *Escherichia coli* and *Staphylococcus aureus* was performed by the agar diffusion and the microdilution methods; and by agar diffusion method against *Candida albicans, Candida parapsilosis, Candida glabrata* and *Candida krusey*. The fruits extract showed inhibition of bacterial growth for *E. coli* and *S. aureus* with a bacteriostatic effect on *E. coli* and a bactericidal effect on *S. aureus*. There was no effect on any *Candida* species. Ethanolic extract of *G. americana* fruits seemed to be a promising source of phytotherapic drugs with a spectrum of action on diverse microorganisms in non-toxic concentrations.

Key words: Alphaherpes virus, medicinal plant, antimicrobial, antiviral, cell line, broad spectrum.

INTRODUCTION

In several countries, for generations, medicinal plants have been widely used. However, it has been only for the last two decades that plant as a medicine has been integrated in allopathic therapies in public health assistance (Santos et al., 2011). Easy access and low cost of natural origin products should be recommended

as a therapeutic source, mainly in low-income communities (Brasileiro et al., 2008). However, the plants used in any herbal preparation require strict quality control since it may exhibit toxic and/or variable chemical composition. Thus, their toxicity should be checked beforehand. The current toxicology methods employed for the initial determination of toxicity or screening of these substances are *in vitro* assays using cell cultures, the so-called cytotoxicity assays. The toxicity may be assessed via several forms such as morphology, incorporation of stains or radioactive nucleotides. These methods are increasingly being used to replace the animal testing together with their validation, standardization and reproducibility (Eisenbrand et al., 2002; Cos, 2006; Rispin et al., 2006).

Investigation and establishment of new compounds as a basis for medicine and/or drugs remains essential and necessary, despite the control and effective treatment of many diseases. For this, an increasing number of phyto-studies have found molecules of biological and/or therapeutic potential (Rates, 2001; Clardy and Walsh, 2004; Balunas and Kinghorn, 2005; Cos, 2006). This is especially so in the field of veterinary medicine, with the rise in the interest about the use of the medicinal plants in recent decades, which resulted in scientific studies to corroborate natural products recommendations (Viegi et al., 2003; Pieroni et al., 2006; Martínez and Luján, 2011) which turns the study involving animals pathogens of great interest.

Polymicrobial diseases, which involve two or more microorganisms that act synergistically, or in succession, mediating complex disease progress are a point of concern. This is because intermicrobial interactions and environmental cues determine infection outcomes, such that specific microbial populations under certain conditions may enhance or predict disease progression (Bakaletz, 2004; Peters et al., 2012). Therefore anti-microbial agents can act directly on the micro-organisms without affecting normal host cell and with a selective toxicity independent if they are synthetic or derived from plants (Cos, 2006; Samy and Gopalakrishnakone, 2010). Moreover, the synthetic drugs lead to emergence of resistance strains. Thus, medicinal plants would be an alternative to overcome this and they could either substitute or complement synthetic drugs (Chattopadhyay and Naik, 2007; Son et al., 2013).

These novel natural substances may be tested through *in vitro* bioassays that provide rapid and effective mechanisms of screening (Houghton, 2000; Cos, 2006; Samy and Gopalakrishnakone, 2010). Regardless of which bacterial, yeast, or viruses are the target of this search, concomitant or previous assays of the toxicity of these compounds should be carried out to guarantee and provide a safe and effective use, and consequent safety and reliability (Houghton, 2000; Eisenbrand et al., 2002; Cos, 2006).

Studies on the Rubiaceae family demonstrated a variety of chemical constituents of medicinal importance. In traditional medicine, several species from this family have been used as bronchitis, asthma, pneumonia, anti-rheumatic, emetic, purgative, diuretic, anti-inflammatory, antiviral and anti oedematogenic agent (Lorenzi and Matos, 2002; Dias, 2013). Specifically, *Genipa americana*, an edible fruit tree, besides being used for comfitures and alcoholics beverages, is commonly used in traditional medicine to treat anemia, jaundice, asthma, and liver and spleen diseases (Lorenzi and Matos, 2002; Moreira et al., 2002). *In vitro* studies of *G. americana* fruit showed a broad spectrum antibacterial activity (Barbosa, 2008) and an interference on trophoblast-like cells proliferation but not on differentiation (Conceição et al., 2011). Thus, it is prudent to investigate this much used species for its *in vitro* toxicity and to evaluate its anti-viral and anti-microbial potential.

MATERIALS AND METHODS

G. americana L. (Rubiaceae) branches, fruit and leaves were collected through botanical field work in January 2006, in Rio de Engenho district, Ilhéus, Bahia, Brazil. Plant habitat was characterized by argillaceous ground, a humidified environment, and cacao culture, in the Brazilian northeastern hieroglyphic forest (Mata Atlantica forest). Plant collection coordinates were 14° 51' S and 39° 04' W. The voucher specimen was identified and deposited in the herbarium of the Universidade Estadual de Santa Cruz, under identification number of HUESC13.923. The study was conducted following Brazilian access to nature law (permission number: IBAMA no. 02001.001749/2012-31).

Preparation of ethanolic extracts

Plant material was dried for 8 h at 28°C protected from the light. Dry plant material was reduced to a powder with mechanical knifes. A 10 g sample of dried and powdered plant material was macerated in 100 mL ethanol for 24 to 48 h through direct contact with solvent and with mechanical agitation. The marc was filtered through Whatman number 1 filter paper and evaporated to dryness under reduced pressure. Each plant material was soaked in recovered solvent once more to make a new extraction and further lyophilized. For bioassays, firstly a stock solution of each extract was made diluting 100 mg in 100% sterile dimethyl sulfoxide (DMSO Sigma®). Then, this stock solution was diluted in Minimum Eagle Medium(MEM, Atená®) to yield concentrations of 4, 1 and 0.8 mg/mL, respectively of fruits (FE), branches (BE) and leaves (LE)

*Corresponding author. E-mail: aoconceicao@uesc.br.

ethanolic extracts. These extracts were filtered with 10 µm pore filter, distributed in aliquots, and stored at -20°C.

Cell line

The Vero cell line from green monkey kidney (ATCC CCL-81) was kindly provided by Instituto Butantan. The cells were maintained in MEM with fetal bovine serum 8% in 37°C incubator, 5% CO_2. For the experiments, cells were subcultured on the previous day in sterile and disposable 96-microplates (3.0×10^4 cells/well).

Viruses

The suid alphaherpesvirus type-1 (SuHV-1) EMBRAPA: BRMSA 3, 00588 and equid alphaherpes virus type-1 (EHV-1) A4/72 Brazilian strains were used in this study.

Cytotoxicity assay

The morphological method was applied here. Extracts diluted in MEM (two base) from their stock solutions were added to the cell microplates. The changes or cell death were visualized at inverted light microscope every day up to 72 h using as a parameter, the control (cells without extract). The first concentration of the extract that did not generate cell morphological changes was called the maximum non-cytotoxic concentration (MNCC) and it was used in antiviral assays.

Antiviral assay

The antiviral activity of the extracts was based on the inhibition of cytopathic effect and consequent reduction of viral titer (Barros et al., 2012). Extracts were considered positive and promising when the viral titer of treated (extracts) and infected cells had a statistical significant reduction in the viral titer as compared to control infected cells (without addition of the extracts). Foscarnet (Sigma-Aldrich, Brazil) at 200 µg/mL was used as control. The statistical difference between treatments was accessed by *t* test considering 95% confidence interval ($p<0.005$).

Antibacterial and antifungal assays

For this study, American Tissue Culture Collection (ATCC) bacteria (*E. coli*, INCQS-00325-ATCC-35218; *S. aureus* INCQS-00015-ATCC-25923) and yeast (*C. albicans*, ATCC 14057); *C. krusey*, ATCC 6258; *C. parapsilosis*, ATCC 22018; and *C. glabrata*, ATCC 2301) were used. Bacteria were obtained from Collection of Reference Microorganisms on Health Surveillance (CMRVS, FIOCRUZ-INCQS), Rio de Janeiro, Brazil. Yeasts were kindly provided by Dr. Sydney Hartz Alves from Mycological Research Laboratory (LAPEME) at Federal University of Santa Maria. These micro-organisms were of clinical interest.

Agar gel diffusion (AGD) and microdilution techniques were used for both antibacterial and antifungal evaluation. For AGD, the well variant was used. For that, bacterial and yeast inoculums were prepared at an equivalent of 0.5 to 1.0 of McFarland scale (approximately cell density of 1.5×10^8 CFU/mL) and spread with sterile swab all over the Petri dishes containing nutrient or Sabouraud dextrose agar. Solutions of 0.125, 0.25, 0.5 and 1, 2.5, 5 and 10 mg/mL were prepared with NaCl 0.9% solution immediately before the test. After bacterial and yeast culture in Petri dishes, holes with 6 mm diameter were done in the agar where 50 µL of samples and controls were applied. Controls consisted of chloramphenicol (50 µg/ml), ketoconazole (50 µg/mL), chlorexidine (1%) and NaCl 0.9%. The plates were incubated overnight at 37°C in a humidified chamber to avoid evaporation of the medium. All tests were repeated at least three times.

To analyze the antibacterial activity of the samples, inhibition halo of ≥ 14 mm at ≤ 100 mg/mL were used as criteria to consider a sample as antibacterial. To analyze the antifungal activity, inhibition halo ≥ 10 mm was used as criteria to consider the extract as antifungal (Pedroso et al., 2014).

For microdilution technique, a serial dilution (10^{-1} to 10^{-9}) of both bacteria was done in brain heart infusion broth (BHI). After 24 h at 37°C, 10 µL of the serial dilutions were plated in nutrient agar plates and incubated at 37°C for another 24 h. Thereafter, bacterial colonies were counted. To perform antibacterial test, the latest dilutions having ≥100 CFU were chosen (*E. coli* = 10^{-7}; *S. aureus* = 10^{-5}). For that, 100 µL of extract and 100 µL of bacterial inoculum were distributed in 96 well plates in triplicates. Controls consisted of chloramphenicol (100 µL CHLOR+ 100 µL inoculum), inoculum (100 µL inoculum+100 µL BHI), plant extracts (100 µL extract+100 µL BHI) and BHI (200 µL) only. Extracts were prepared from 10 mg/mL starting solution and diluted in BHI giving final solutions of 0.125, 0.5 and 1.0 mg/mL. After 24 h incubation at 37°C, the optical density was measured by spectrophotometry at 450 nm wave length to determine the minimal inhibitory concentration (MIC). Background was reduced subtracting optical density of plant extract without inoculum and BHI only. Then, the subculture was done in nutrient agar to visualize bacterial growth and determine the minimal bactericidal concentration (MBC).

RESULTS AND DISCUSSION

The initial focus of this study was to investigate the *in vitro* toxicity of extracts of *G. americana* prior to antiviral assay. Here, the evaluation was based on cellular morphological changes where higher MNCC values mean less cytotoxicity, and lower value means the greater cytotoxicity. From the cellular morphological alterations caused by extracts and visualized at inverted cell microscope, the maximum non-toxic concentration of each extract (MNCC) was determined. The MNCCs of FE and BE extracts were of 0.5 and 0.25 mg/mL, respectively. The LE extract showed none cytotoxicity in all tested dilutions. Thus, the concentration of 0.2 mg/mL was established for use in antiviral assays. The toxic alteration on cell monolayer ranged from cell death with total monolayer destruction to formation of vacuoles and cellular condensation (Figure 1). While all extracts presented low cytotoxicity on Vero cells, special attention should be paid to FE extract which contributes to considering *G. americana* fruit extract as a promising phytotherapic agent.

Antiviral therapy is more effective on human herpes viruses, nevertheless the animal herpes viruses can serve as models and to better understand this family of virus through *in- vitro* and *vivo* experiments (Field et al., 2006; Son et al., 2013). In the case of equine alphaherpes virus infections, the use of antivirals combined with other measures is beneficial and well-

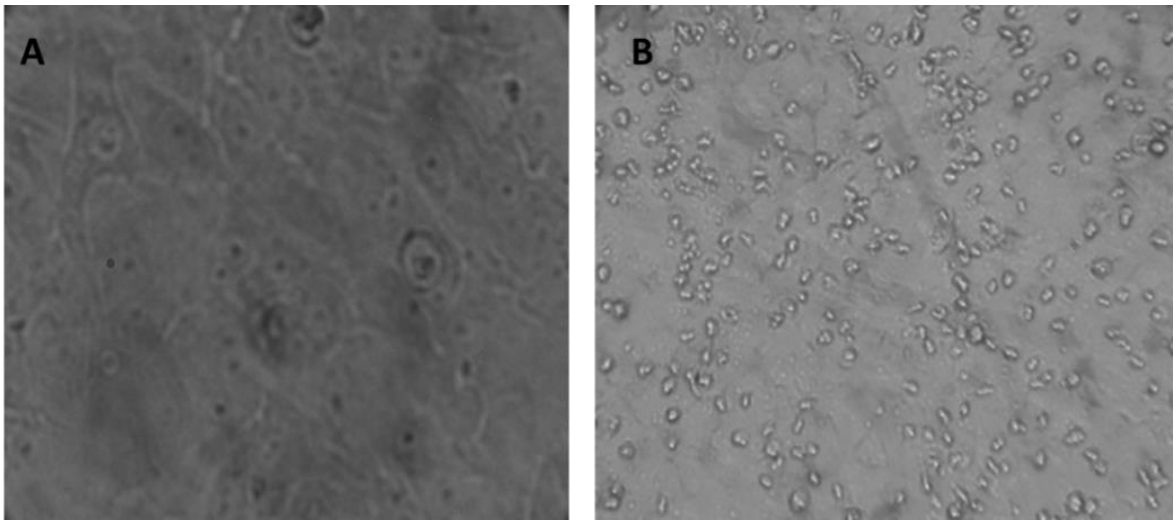

Figure 1. Cytotoxic evaluation of *G. americana* on Vero cells. A) Cell control (without extract); B) Cells with ethanolic extract of *G. americana* fruit (FE) at 4 mg/mL. Image obtained with inverted light microscope at 200x.

Table 1. *G. americana* extracts antiviral activities against suid (SuHV-1) and equine (EHV-1) alphaherpes viruses.

Ethanolic extracts	Viral inhibition (%)	
	EHV-1	SuHV-1
Branches (BE)	0	79
Fruits (FE)	86*	0
Leaves (LE)	0	73
Foscarnet (0.2 mg/mL)	0	99*

*p<0.005 by *t* test.

studied (Garré et al., 2007; Vissani et al., 2016).

The antiviral evaluation of the extracts is described in Table 1. Only FE showed a significant inhibition to EHV-1, around 90% (p< 0.005). The LE and BE extracts were not inhibitors. On the other hand, BE and LE extracts inhibited 79 and 73% of SuHV-1 effect, respectively, while FE demonstrated any antiviral activity to SuHV-1. The Foscarnet, synthetic antiviral agent, was used as positive control at non-toxic concentration of 0.2 mg/mL. The strain of EHV-1 was shown to be resistant to it while the SuHV-1 strain was susceptible with a reduction of 2 log in its viral titer equivalent to 99% of inhibition. Garré et al. (2007) studying diverse nucleoside analogues and foscarnet also verified that this last is not appropriate for treatment of infected horses because it was the least effective and with differences between the isolates.

The suid alphaherpes virus is widely studied as a model for herpes virus biology, but there are diverse studies on antivirals from medicinal plants aiming in the future to decrease the infection in addition to vaccination and the negative impact of this disease on pig industry (Pomeranz et al., 2010; Son et al., 2013). For suid alphaherpes virus, less intensive antiviral activity was seen with the BE and LE extracts. In contrast, the foscarnet was active against this virus with 99% of inhibition. These results reinforce the differences between animal viruses' models.

The procedure to study antibacterial and antifungal agents focusing on their growth can be done with different methods (Cos, 2006). Here, we used qualitative and quantitative techniques against two important Gram negative and positive bacteria. Ethanolic extracts from *G. americana* leaves, brunch and fruit showed variability between the two types of bacterial used. *E. coli* and *S. aureus* were resistant to both leaves and brunch extracts. Conversely, *E. coli* and *S. aureus* was shown to be sensitive to FE extract (Table 2 and Figure 2). Through AGD halo of inhibition was formed at 1 and 0.5 mg/mL, for *E. coli* and *S. aureus*, respectively. Through MIC/MBC, a bacteriostatic effect of *E. coli* and bactericidal effect of *S. aureus* at ≥0.05 mg/mL was remarked.

The antibacterial action against *E. coli* at ≥ 1 mg/mL (Table 2) differed from negative results found by Gonçalves et al. (2005). We consider our results promising, since the AD technique is more sensitive than disc impregnation used by Gonçalvez et al. (2005). It was also possible to establish a dose dependent response, and this technique is less susceptible to intrinsic errors. Besides, the results was shown to be still more promising with MIC and MBC techniques since bactericidal effect of ethanolic extract from *G. americana* fruit was clearly seen.

In conclusion, due to the presence of steroids found in a prior study (Conceição et al., 2011) and literature report

Table 2. Antibacterial activity of ethanolic extract of *G. americana* by agar diffusion technique against *E. coli* and *S. aureus*.

mg/mL	*E. coli* (ATCC-35218)			*S. aureus* (ATCC-25923)		
	LE	BE	FE	LE	BE	FE
10	R	R	14	ND	ND	18
2.5	R	R	13	ND	ND	14
1	R	R	13	R	R	11
0.5	R	R	R	R	R	15
0.25	R	R	R	R	R	R
0.125	R	R	R	R	R	R
CHLOR		40			39	

LE– leaves; BE– brunch; FE– fruit; CHLOR- chloramphenicol. Inhibition zone diameter is expressed in millimeters (mm). The results represent the media of two to four experiments. R– resistant; ND– not done.

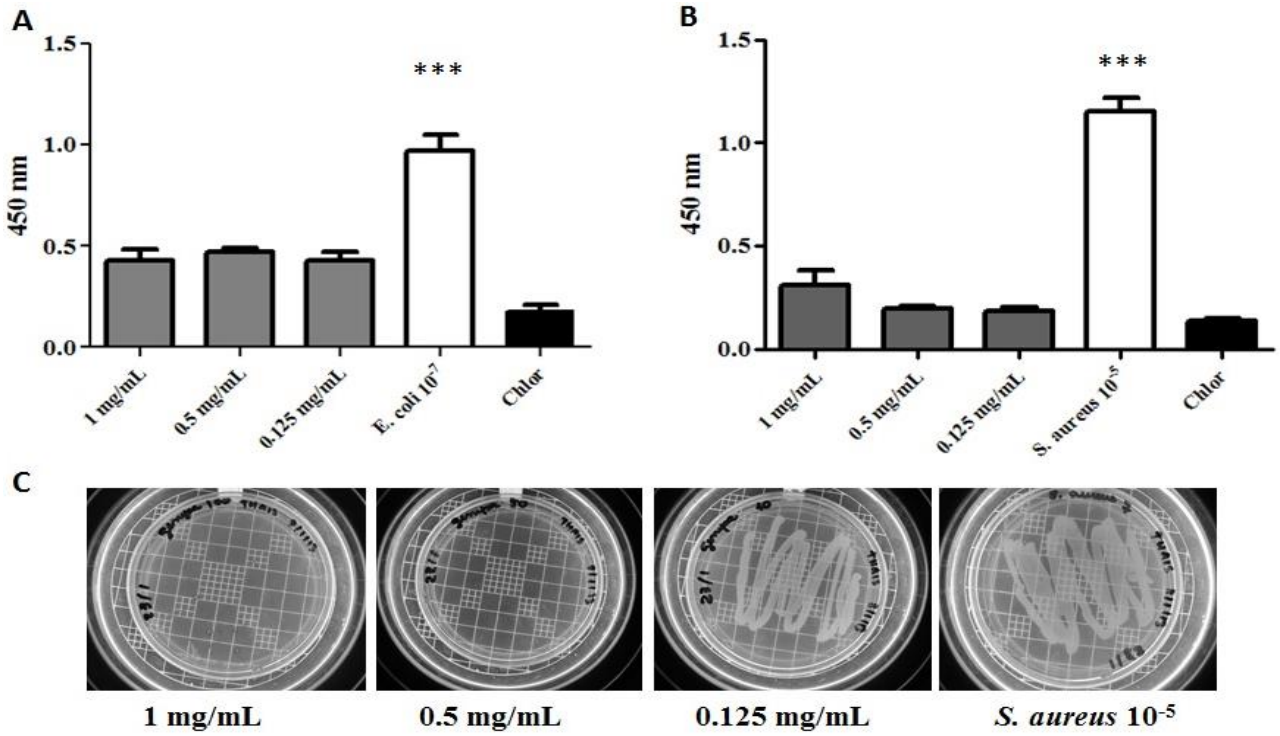

Figure 2. Minimal inhibitory concentration (MIC) (A and B) and minimal bactericidal concentration (MBC) (C) of *G. americana* fruit ethanolic (FE) extract against *E. coli* (A) and *S. aureus* (B, C).*** p<0.01 by Anova followed by Tukey test.

related to antimicrobial activity (Taleb-Contini et al., 2003), it seems that steroids found in *G. americana* fruit ethanolic extract play an important role in microbial fight. Hence, this work emphasizes the importance of studies on medicinal plants with the purpose of obtaining no cytotoxic new molecules with biological and therapeutic potential, useful for the prevention or treatment of polymicrobial diseases.

Conflict of interest

All the authors have not declared any conflict of interest.

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REFERENCES

Bakaletz LO (2004). Developing animal models for polymicrobial diseases. Nat. Rev. Microb. 2:552-568.

Balunas MJ, Kinghorn AD (2005). Drug Discovery from medicinal plants. Life Sci. 78:431-441.

Barbosa DA (2008). Avaliação fitoquímica e farmacológica de Genipa americana L. (Rubiaceae). Master dissertation, Universidade Federal do Rio de Janeiro, Brazil.

Barros AV, Araújo LM, Oliveira FF, Conceição AO, Simoni IC, Fernandes MJB, Arns CW (2012). *In vitro* evaluation of the antiviral potential of Guettarda angelica against animal herpesviruses. Acta Sci. Vet. 40(4):1068.

Brasileiro BG, Pizziolo VR, Matos DS, Germano AM, Jamal CM (2008). Medicinal plants used by the population assisted by the "Programa de Saúde da Família" (Family Health Program) in Governador Valadares County - MG, Brazil. Braz. J. Pharmac. Sci. 44(4):629-636.

Chattopadhyay D, Naik TN (2007). Antivirals of ethnomedicinal origin: structure-activity relationship and scope. Mini-Rev. Med. Chem. 7:275-301.

Clardy J, Walsh C (2004). Lessons from natural molecules. Nature 432:829-837.

Conceição AO, Rossi MH, Oliveira FF, Takser L, Lafond J (2011). *Genipa americana* (Rubiaceae) fruit extract affects mitogen-activated protein kinase cell pathways in human trophoblast–derived BeWo cells: implications for placental development. J. Med. Food 14(5):483-494.

Cos P, Vlietinck AJ, Vanden Berghe D, Maes L (2006). Anti-infective potential of natural products: How to develop a stronger *in vitro* 'proof-of-concept'. J. Ethnopharmacol. 106:290-302.

Dias SRK, Mendonça ACAM, Silva MAP (2013). Ethnobotanical, phytochemical and pharmacological aspects Rubiaceae species in Brazil. Rev. Cubana Plant. Med. 18(1):140-156.

Eisenbrand G, Pool-Zobel B, Baker V, Balls M, Blaauboer BJ, Boobis A, Carere A, Kevekordes S, Lhuguenot JC, Pieters R, Kleiner J (2002). Methods of *in vitro* toxicology. Food Chem. Toxicol. 40:193-236.

Field HJ, Biswas S, Mohammad IT (2006). Herpesvirus latency and therapy-From a veterinary perspective. Antivir. Res. 71: 127-133.

Garré B, Van Der Meulen K, Nugent J, Neyts J, Croubels S, De Backer P, Nauwynch H (2007). In vitro susceptibility of six isolates of equine herpesvirus 1 to acyclovir, ganciclovir, cidofovir, adefovir, PMEDAP and foscarnet. Vet. Microbiol. 122:43-51.

Gonçalves AL, Alves Filho A, Menezes H (2005). Comparative study on antimicrobial activity of some native tree extracts. Arq. Inst. Biol. 72(3): 353-358.

Houghton PJ (2000). Use of small scale bioassays in the discovery of novel drugs from natural sources. Phytother. Res. 14:419-423.

Lorenzi H, Matos FJA (2002). Plantas medicinais no Brasil: nativas e exóticas. Lorenzi, H, Matos, FJA (eds.), Instituto Plantarum, São Paulo. 512p.

Martínez GJ, Luján MC (2011). Medicinal plants used for traditional veterinary in the Sierras de Córdoba (Argentina): An ethnobotanical comparison with human medicinal uses. J. Ethnobiol. Ethnomed. 7(1): 23.

Moreira RCT, Costa LCB, Costa RCS, Rocha EA (2002). Ethnobotanical approach on the use of medicinal plants in Vila Cachoeira, Ilhéus, Bahia, Brasil. Acta Farm. Bonaerense 21(3):205-211.

Peters BM, Jabra-Rizk MA, O'May GA, Costertone JW, Shirtliff ME (2012). Polymicrobial Interactions: Impact on Pathogenesis and Human Disease. Clin. Microbiol. Rev. 25(1):193-213.

Pieroni A, Giusti ME, Pasquale C, Lenzarini C, Censorii E, Gonzáles-Tejero MR, Sánchez-Rojas CP, Ramiro-Gutiérrez JM, Skoula M, Johnson C, Sarpaki A, Della A, Paraskeva-Hadijchambi D, Hadjichambis A, Hmamouchi M, El-Jorhi S, El-Demerdash M, El-Zayat M, Al-Shahaby O, Houmani Z, Scherazed M (2006). Circum-Mediterranean cultural heritage and medicinal plant uses in traditional animal healthcare: a field survey in eight selected areas within the RUBIA project. J. Ethnobiol. Ethnomed. 2:16.

Pomeranz LE, Reynolds AE, Hengartner CJ (2005). Molecular biology of pseudorabies virus: impact on neurovirology and veterinary medicine. Microbiol. Mol. Biol. Rev. 69(3):462-500.

Rates SMK (2001). Plants as source of drugs. Toxicon, 39:603-613.

Rispin A, Stitzel K, Harbell J, Klausner M (2006). Ensuring quality of in vitro alternative test methods: Current Practice. Regul. Toxicol. Pharm. 45:97-103.

Samy RP, Gopalakrishnakone P (2010). Therapeutic potential of plants as anti-microbials for drug discovery. eCAM 7(3):283-294.

Santos RL, Guimaraes GP, Nobre MSC, Portela AS (2011). Analysis about phytotherapy as an integrating practice in the Brazilian Unified Health System (UHS). Rev. Bras. Pl. Med. 13(4):486-491.

Son M, Lee M, Sung GH, Lee T, Shin YS, Cho H, Lieberman PM, Kang H (2013). Bioactive activities of natural products against herpesvirus infection. J. Microbiol. 51(5):545-551.

Taleb-Contini SH, Salvador MJ, Watanabe E, Ito IY, Oliveira DCR (2003). Antimicrobial activity of flavonoids and steroids isolated from two Chromolaena species. Rev. Bras. Cienc. Farm. 39(4):403-408.

Viegi L, Pieroni A, Guarrera PM, Vangelisti R (2003). A review of plants used in folk veterinary medicine in Italy as basis for a databank. J. Ethnopharmacol. 89:221-244.

Vissani MA, Thiry E, Dal Pozzo F, Barrandeguy M (2016). Antiviral agents against equid alphaherpesviruses: Current status and perspectives. Vet. J. 207:38-44.

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Full Length Research Paper

Inhibition of aflatoxin B_1 production of *Aspergillus flavus* isolated from peanut seeds using *Lycium halimifolium* Mill leaves fractions

Camélia Mosbah[1,2]*, Asma Milet[1], Mounira Kara Ali[1], Imen Talhi[1] and Noureddine Kacem Chaouche[1]

[1]Laboratory of Mycology, Biotechnology and Microbial Activity (LaMyBAM), Brothers Mentouri University, Constantine, Algeria.
[2]Department of Biology, Faculty of Nature and Life Sciences, Larbi Ben M'hidi University, Oum el Bouaghi, Algeria.

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The aim of this study is to explore the impact of *Lycium halimifolium* Mill leaves extracts on mycotoxin B_1 production *in vitro,* as well as the inhibitor effect of the aflatoxin B_1 synthesis by *Aspergillus flavus* strain. The strain was isolated, identified (gi |146746162 | EF409803.1.) and positively tested for the production of Aflatoxin B_1. The antimycotoxinogenesis effect was investigated using *L. halimifolium* Mill leaves consisting of butanol and ethyl acetate fractions. Indeed, the fractions were tested to stop the secretion of aflatoxin B_1 by *A. flavus* in submerged culture (YES), simultaneously. The growth of the strain was evaluated as biomass response. The antimycotoxinogenesis activity showed that the two fractions: ethyl acetate and butanol of *L. halimifolium* Mill leaves were able to inhibit significantly the synthesis of mycotoxin AFB_1. Thus, the secretion inhibition percentage was calculated in comparison with the control, using HPLC-PDA technique, and estimated at 96.83% for butanolic fraction and 94.99% for ethyl acetate fraction. To verify its correlation with biomass, the growth inhibition percentage was also estimated. The results showed that the dry weight concentration under butanolic and ethyl acetate fractions was 1.6 and 2 g/l, respectively, corresponding to 65.22 and 56.53% of inhibition growth percentage.

Key words: Aflatoxin B_1, antimycotoxinogenesis activity, high performance liquid chromatography coupled with photodiode array detector (HPLC-PDA), natural extract.

INTRODUCTION

Mycotoxins are low molecular weight compounds that are synthetized during secondary metabolism by fungi especially those belonging to the genus *Aspergillus*, *Penicillum* and *Fusarium* (Streit et al., 2012). Aflatoxins are a group of toxic metabolites, called mycotoxins, produced by different species of toxigenic fungi such as

*Corresponding author. E-mail: kamimosbah@yahoo.fr. Tel: 0021364907291. Fax: 00213 31426102.

Aspergillus flavus, *Aspergillus parasiticus, Aspergillus nomius* (Huwig et al., 2001). Among the different types of aflatoxins identified, the major groups are aflatoxin B_1 (AFB_1), B_2 (AFB_2), G_1 (AFG_1), G_2 (AFG_2), M_1 (AFM_1) and M_2 (AFM_2). The biosynthesis of aflatoxins is strongly dependent on chemical and physical growth conditions, that is, substrate composition, temperature, pH, water activity and the particular combination of different parameters that can be completely inhibited or activated by the biosynthesis of these aflatoxins (Giorni et al., 2008). Moreover, the biosynthesis of aflatoxin is established by the conversion of acetate to norsolorinic acid (NOR) by synthases (FAS) and a polyketide synthase (NR-PKS, PksA) which are involved in the synthesis of the polyketide from a hexanoyl (the initial substrate for aflatoxin formation (Trail et al., 1995; Crawford et al., 2008). Aflatoxin B_1 (AFB_1) is the most potent of the known AFs. It was classified within class 1 of human carcinogens especially the hepatocellular carcinoma (Brown, 2009) and considered as potent mutagenic. That caused the damage of cells by two different ways; firstly, AFB_1 ($C_{17}H_{12}O_6$) is activated to AFB_1 -8,9-epoxide and forms adduct primarily at N7 position of guanine. In addition , the AFB_1 -8,9-epoxide may interfere with the normal functioning of the nerve cells by forming DNA adducts, protein adducts, oxidative stress factors, mitochondrial directed apoptosis of the nerve cells as well as inhibiting their synthesis of protein, RNA and DNA (McGlynn et al., 2003); secondly, it is a potential immunosuppressive agent which several human studies have reported that aflatoxins cause an increase in circulating alpha tumor necrosing factor suggesting that these mycotoxins are immunotoxic in humans. AFB_1 -8,9-epoxide affects also other organs and tissues, such as the lungs, kidney, heart damage and the entire respiratory system (Wagacha and Muthomi, 2008).

Fungi are generally controlled by synthetic fungicides; however, the use of these is increasingly restricted due to the harmful effects of pesticides on human health and the environment (Hermiche et al., 2012). Thus, there are a growing interest on the research of possible use of the plant extracts for control of the pest and diseases in agriculture which is less harmful to one's health and environment (Logardia, 2012). A large number of compounds, extracts and essential oil from natural sources including medicinal plants such as roots, leaves, seeds and flowers have been reported to inhibit the production of aflatoxin (Paranagama et al., 2003; Benariba et al., 2013; Martins et al., 2014). The negative impacts of aflatoxins on human health led to the investigation of strategies to minimize, inactivate or eliminate the effects of these toxins in contaminated products. In this context, natural sources such as medicinal plants were studied for its therapeutic effect vis-à-vis aflatoxins. Among them, the genus *Lycium* (Solanaceae family) has been identified as a rich source of antioxidant compounds (Donno et al., 2014). This genus was used as a hedge plant and windbreak; the dry powder of its leaves was used as a protector from eye diseases (Potterat, 2010). In addition, *Lycium halimifolium* Mill is considered as a healthy food (Amagase and Farnsworth, 2011). The chemical constituents of *L. halimifolium* Mill are; cyclic peptides, glycoside derived from tryptophan, alkaloids, carotenoids and related compounds (β-caroténe, zeaxanthin dipalmitate, β-cryptoxanthin, lutein), phenolic acids and flavonoids (Quercetin-3-O-rutinoside, Kaempherol-3-O-rutinoside, caffeic acid, vanillic acid) and lipopolysaccharides (LP) (glycoproteins formed by a heteroside and conjugated with a polypeptide chain) (Gu et al., 2007; Stephen et al., 2010; Xiaq et al., 2012; Jin et al., 2013). The recent studies indicate that the extracts of some *Lycium* species possess a range of biological activities, including effects on ageing, neuroprotection, anti-fatigue/endurance, increased metabolism, glucose control in diabetics, glaucoma, anti-oxidant properties, immunomodulation, anti-tumour activity and cytoprotection (Potterat, 2010). To our knowledge, this study represents the first report of the biological properties of *L. halimifolium* Mill isolated from the eastern part of Algeria. The main objective of this work is to isolate and identify a fungi strain, test its antimycotoxinogenesis activity in submerged culture (YES) and evaluate its antifungal efficacy as biomass response.

MATERIALS AND METHODS

The plant material

L. halimifolium Mill plant was harvested from the mountain of Djbel Chettaba situated in Constantine (Eastern Algeria) with latitude 36°19'41.02" and longitude 6°27'4". The samples were collected in spring 2013, corresponding to flowering period. The identification of the plant was established in laboratory of physiology and Ecology, Montouri Brothers University, Constantine, Algeria. The plant leaves were harvested and dried in the shade at room temperature and then were crushed using mortar and pulverized in the mill to obtain powder.

Extraction procedure

For flavonoids extraction, the method of Benhammou et al. (2009) was applied; thus, 20 g of the dry powder of *L. halimifolium* Mill leaves was extracted in 200 mL of methanol for 24h at room temperature. The extracts were concentrated in a rotary evaporator at 60°C under a reduced pressure. The residue was taken with boiling water to dissolve the flavonoids. The aqueous phase containing flavonoids was defatted with petroleum ether and chloroform. The defatted aqueous phase was filtrated through whatman paper No 1; firstly, it was extracted with 100 mL of ethyl acetate to obtain ethyl acetate fraction (ACT). The same operation was executed with 100 mL of n-butanol to obtain butanol fraction (BUT). The extracts were evaporated, weighted and preserved for further use in biological activities. The extraction yield was determined by the following formula:

$$R\% = \frac{mass\ of\ fraction\ residue}{mass\ of\ vegetal\ poweder} \times 100.$$

Microbial material

The isolation of the toxigenic fungus was carried out from the local peanuts of the wilaya of El Taref, Algeria. According to Outtara et al. (2011), a sample (50 g) of fifty peanut seed from the crop year 2013 was moistened with sterile distilled water and left at room temperature in Petri plates during 7 days for the development of mycelium. The purification of the interest strain (*Aspergillus*) was made by successive subcultures on the PDA medium which allows the isolation of pure strain. Fungal isolate was characterized macroscopically and microscopically according to Pitt and Hoking (2009). This characterization was performed in the Laboratory of Mycology, Biotechnology and Microbial Activity (LaMyBAM). The molecular identification of fungal isolate was performed at the Laboratory of Microorganisms and Active Biomolecules (LMBA), Tunisia. The used primers for the PCR amplification were the universal primers ITS1, ITS4 for the ITS gene (Josefa et al., 2004). The purification of the PCR products was achieved using the horizontal agarose gel of ethidium bromide. The amplified genes were sequenced using the same primers sited above and the sequences were corrected by the Bio-edit program and deposited in Genbank data base. The DNA sequences were compared to those previously published in Genbank using the BLASTN program.

Antimycotoxinogenesis activity of *L. halimifolium* M. leaves fractions

Mycotoxinogenesis activity

In order to demonstrate the aflatoxinogenesis of this isolate, a fermentation was established; thus, 50 mL of YES liquid medium consisting of g/L: yeast extracts (20), sucrose (150), $MgSO_4.7H_2O$ (0.5), $ZnSO_4.7H_2O$ (0.01), $CuSO_4.5H_2O$ (0.005) (Pamel et al., 2011) was placed in 250 mL flasks, and autoclaved at 120°C for 15 min. The inoculation was performed by adding 1mL of a spore suspension (10^5 spores/mL) from interest strain youth culture. The flasks were incubated for 14 days at 25°C with intermittently manual agitation.

Detection of aflatoxin B_1

After the incubation period, the flask content was filtered through Whatman No.1. The mycelia obtained were placed on preweighed Petri plates and dried at 50°C for 6 h and then at 40°C over night. The net dry weight of mycelia was determined. In addition, the filtrate was used to detect the presence of mycotoxins especially, aflatoxin B_1. Thus, 50 mL of the filtrate was added to 100 mL of chloroform. The mixture was thoroughly stirred for 10 min and allowed to settle. This operation was repeated successively by adding 50 mL, and then 30 mL of the chloroform to the aqueous phase. The chloroform extract obtained was filtered through Whatman No. 01 and concentrated by evaporation under vacuum using a rotary evaporator until a volume of 2 mL, to constitute the aliquot of mycotoxin crude (AMC) (Multon, 1982). 5 µL of AMC was spotted on silica gel plate (silica gel 60 F254) thin layer chromatography, on the same line where 5 µL of Aflatoxin 1 control (NOVAKITS with 99.7% purity, product code: STD-AFB_1 -P1) was deposited. The plate was placed in a chromatography tank and dipped in an eluting solvent mixture consisting of toluene-ethyl acetate- formic acid (5:4:1), respectively. After the migration, the plate was examined under a UV lamp at a wavelength of 365 nm. The presence of aflatoxin B_1 was characterized by a blue fluorescence spot with the same R_f as the control. All tests were performed in three replicates (Dutton et al., 1985).

The presence of aflatoxin B_1 was confirmed using high performance liquid chromatography coupled with photodiode array detector (HPLC-PDA). The HPLC-PDA consisted of Waters e2695 separation Modules equipped with a Photodiode Array Detector (PDA), Gradient Pump (with four pumps), automatic injector, tiny computer with software Empower 3, control and a detector (UV VIS,with a C18 guard column (Macherynagel 250/4.6 nucleodur); the column temperature was between 5 to 40°C. To constitute the mobile phase of mycotoxins, a gradient solvent system with solvent A (10 mL/L of Trifluoroacetic acid, TFA) and solvent B (acetonitrile) was used; however, the initial percentage of solvent B was 10%, which was raised to 50% in 30 min, then 90% in 4 min, lowered to 10% in 2min, and held at 10% at a flow rate of 1mL/min. The injection volume was 20 µL for sample extract and the detector was operated at wavelength of 254 nm (Frisvad, 1983).

Anti mycotoxinogenesis activity

To study the effect of two fractions of *L. halimifolium* Mill leaves, extracted previously (ACT and BUT) as inhibitors of aflatoxin B_1 synthesis by interest isolate, the flasks of 250 mL contained 50 mL of YES liquid medium. Three flasks were supplemented with 1mg of ACT fraction; three other flaks were added to 1mg of BUT and the last three flasks were kept without treatment and served as control group. All the flasks were inoculated with 1mL of a spore suspension (10^5spores/mL) from an interest youth strain culture. The flasks were incubated for 14 days at 25°C with intermittently manual agitation.

RESULTS AND DISCUSSION

This work is based on the study of antimycotoxinogenesis effect of substances extracted from leaves of *L. halimifolium* Mill, and the focus of attention is the inhibition of Aflatoxin B_1 synthesis by *A. flavus*.

Determination of extraction yield

Two fractions, namely, ethyl acetate (ACT) and butanol (BUT) were obtained through the applied extraction protocol, and the extraction yield was calculated. It was found that the butanol fraction had the greatest yield with a rate of 28.5%, followed by the ethyl acetate fraction with a rate of 10.08%. The variation of extraction levels may be due to several factors including the interaction with the environment (type of climate, soil, period of harvesting and extraction method).

Characterization and identification of the interest strain

The macroscopic aspect of the colonies' morphology showed that this isolate has very rapid rate of growth (three days), characterized by a powdery texture, with yellow-green to olive green color fronds and may have a white border, denser toward the center. The reverse has cream yellowish color in PDA medium (Figure 1a). Otherwise, the microscopic aspect of the interest strain revealed septed hyphae with rather long conidiophores which have a rather rough texture. The vesicles are spherical to elongate, metulae cover the vesicle from

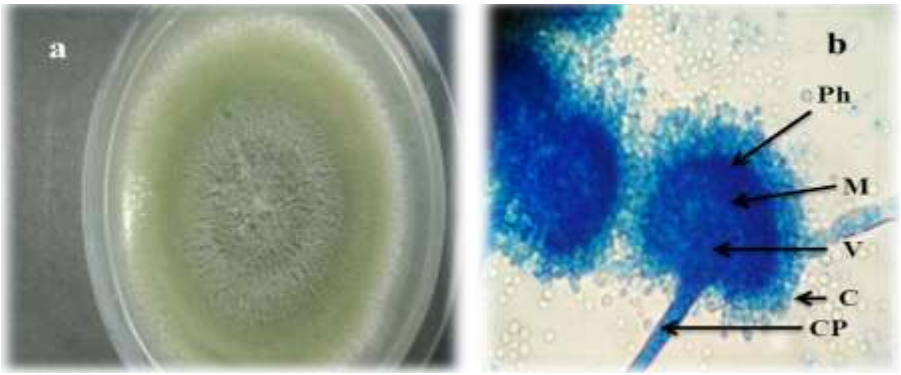

Figure 1. Morphology of interest stain: (a) Macroscopic aspecton PDA medium; (b) microscopic aspect at 100x magnification. CP: conidiophore, C: conidia, V: vesicle. M: Metulae, Ph: phialide.

AAGATTGATTTGCGTTCGGCAAGCGCCGGCCGGGCCTACAGAGCGGGTGACAAAGCCCCATACGC
TCGAGGATCGGACGCGGTGCCGCCGCTGCCTTTGGGGCCCGTCCCCCCCGGAGAGGGGACGACGA
CCCAACACACAAGCCGTGCTTGATGGGCAGCAATGACGCTCGGACAGGCATGCCCCCCGGAATAC
CAGGGGGCGCAATGTGCGTTCAAAGACTCGATGATTCACGGAATTCTGCAATTCACACTAGTTATC
GCATTTCGCTGCGTTCTTCATCGATGCCGGAACCAAGAGATCCATTGTTGAAAGTTTTAACTGATTG
CGATACAATCAACTCAGACTTCACTAGATCAGACAGAGTTCGTGGTGTCTCCGGCGGGCGCGGGCC
CGGGGCTGAGAGCCCCCGGCGGCCATGAATGGCGGGCCCGCCGAAGCAACTAAGGTACAGTAAAC
ACGGGTGGGAGGTTGGGCTCGCTAGGAACCCTACACTCGGTAATGATCCTTCCGCAGTCACCCTGA
CGGGAAATACCGAG

Figure 2. Final sequences of the ITS gene after been corrected using the "Bioedit" program.

which the phialides were formed; Conidia are globose pale green with smooth to finely roughened walls (Figure 1b).

These results corroborate these obtained by Botton et al. (1990), attesting that this strain belongs to *A. flavus.* The dominance of the genus *Aspergillus* in the contaminating flora grain has also been reported in several studies (Reddy et al., 2010). However, to confirm this identification, the molecular analysis of sequencing *ITS1* and *ITS4* region of nuclear ribosomal operon was compared with available sequences in the GenBank. The sequences were similar to each other (99% similarity) (Figure 2). This comparison showed that this isolate is really *A. flavus*. The ITS gene sequences of the strain were submitted to the GenBank database and the accession number established corresponding to gi |146746162 | EF409803.1.

Antimycotoxinogenesis activity of the extracts of *L. halimifolium* Mill leaves

Mycotoxinogenesis activity

After the incubation period (14 days), the filtrate was utilized to confirm the production of aflatoxin B_1 by the *A. flavus* isolated. This filtrate was added to chloroform, cleaned through Whatman No.1, and concentrated by evaporation under vacuum using a rotary evaporator, to constitute the aliquot of mycotoxin crude (AMC) of control, which was spotted on silica gel plate thin layer chromatography. After the visual observation under UV of this plate, a blue fluorescence color of aflatoxin B_1 was revealed with the same R_f as the control (Figure 3).

The AMC presented many spots with different migration distances, a major spot (R_f= 0, 38) correspond the same spot of aflatoxin B_1 standard (R_f= 0.38).This result confirms the production of aflatoxins by the interest strain *A. flavus*. Several studies have been reported on the mycotoxin contamination of cereals and peanuts which demonstrated that *A. flavus* produced aflatoxin B_1 (Xianwen et al., 2015; Fakruddin et al., 2015).

The presence of aflatoxin B_1 in the filtrate was confirmed by high performance liquid chromatography HPLC (Figure 4), where the AMC has a retention time (Rt) of 30.13 min (Figure 4b) corresponding to the same standard aflatoxin B_1 with retention time (Rt) of 30.01 min (Figure 4a).

Antimycotoxinogenesis activity

In this report, the antimycotoxinogesesis effect of the two

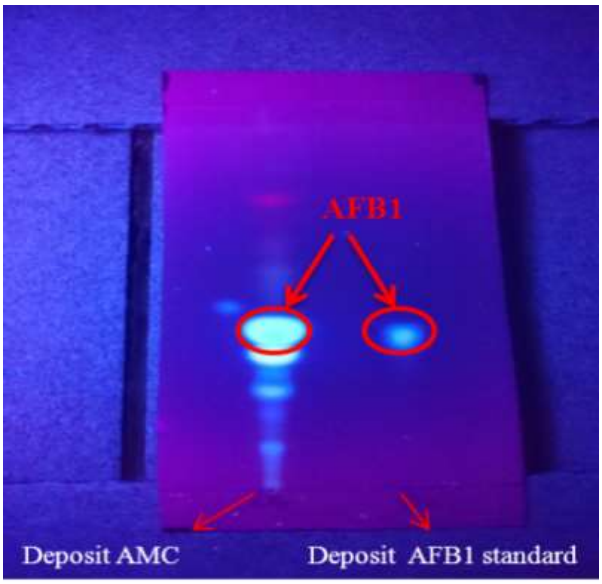

Figure 3. Thin layer chromatography, aliquot of mycotoxin crude of control and aflatoxin B_1 standard.

different fractions (ACT and BUT) of *L. halimifolium* Mill leaves was studied. After the fermentation period, the aliquot of Mycotoxin Crude was spotted in the silica gel plate. The chromatographic profile revealed the presence of a fluorescent blue spot which has an Rf equal to 0.38 only in the control (without fractions) and corresponding to the standard AFB_1, while the two fractions showed a total absence of aflatoxin B_1 (Figure 5).

Our results of TLC demonstrate that the strain *A. flavus* produce aflatoxin B_1, which is consistent with the work of Gacem et al. (2013) who used the same fermentation medium and mobile phase, and he has revealed the presence of a blue fluorescent spot corresponding to the aflatoxin B_1 in the sample of chloroform extract. Another study used a different mobile phase (chloroform/methanol) demonstrated that *A. flavus* can produce a large amount of aflatoxin B_1. Also, Josefa detected Aflatoxins B_1 by TLC and he found that *A. flavus* strains produced aflatoxin B_1 (Josefa et al., 2004).

Also, the antimycotoxinogenesis effect was also confirmed by high performance liquid chromatography HPLC. A remarkable decrease of aflatoxin B_1 synthesis was reported when the growth medium contains the fractions of ethyl acetate and n-butanol (Figure 6); thus, the secretion inhibition percentage was calculated in comparison with the control, using HPLC-PDA technique, and estimated at 96.83% for butanolic fraction (Rt = 30.09 mn) (Figure 6a) and 94.99% for ethyl acetate fraction (Rt = 30.18 min) (Figure 6b).

To verify its correlation with biomass, the growth inhibition percentage was also estimated. The results showed that the concentration of dry weight under butanolic and ethyl acetate extracts was 1.6 and 2 g/L consecutively corresponding to 65.22 and 56.53% of inhibition growth percentage. On the other hand, it is very interesting to note that, the production of aflatoxin B_2 by the strain in the presence of cited fractions (ethyl acetate and butanol fractions) on growth medium, in comparisons with the production of the same toxin by the strain without the presence of fractions, shows a decrease of more than 50% (Figure 6).

However, Neveen et al. (2015) reported that the essential oil of *O. basilicum* has a strong antifungal activity and completely inhibited the growth of *A. flavus* (in the same fermentation medium YES) with a percentage of 70%, while inhibition production of aflatoxin B_1 was observed with a retention time equal to 6.94 minutes for the oil tested against a standard retention time to 6.78 mn. Another study demonstrated that the methanolic extracts of leaves from *Helianthemum scoparium*, *A. schmittianun* and *Daphne gnidium* inhibited the mycelium growth with 65.33, 83.56% and 100% respectively (Mohammedi and Atik, 2013).

Recent studies have demonstrated the antifungal activity of natural compounds extracted from traditional medicinal plants (Mishra et al., 2012). The inhibition growth of *A. flavus* by secondary metabolites (flavonoids and essential oil) has already been reported. These compounds can interfere with biomembranes causing cell damage and causing leakage of cellular materials and finally the death of microorganisms (Dikbas et al., 2008; Abdel Ghani et al., 2008). Considering the large number of different groups of chemical compounds present in extracts of plants (flavonoids, phenols, saponosid, gallic tannins and catechic tannins and alkaloids), it is interesting to note that, the antimicrobial activity is not attributable to a specific mechanism alone but to several targets in the cell.

Plant extracts can completely block the biosynthesis of mycotoxins where fungal growth is not affected (Pusztahelyi et al., 2015). Several investigators studied the identification and application of natural products for inactivation of AFs and reported that some essential oils and other extracts (several flavonoids, and phenolic compounds) of plants could potentially provide protection against AFB1 (Rasooli et al., 2008; Bluma and Etcheverry, 2008). The natural compounds of plant extracts are identified as potential candidates against AFB_1 and essential oils that are able to reduce DNA binding of aflatoxin and phenolic compounds inhibit the biosynthetic pathway of AFB_1 (Alpsoy, 2010). Also the study of Ghorbanian et al. (2008) indicated that inhibition of the aflatoxins synthesis is related to the contact time and the dose of the extract.

This study represents the first report on the evaluation of the antifungal activity and antimycotoxinogenesis effect of *L. halimifolium* Mill leaves. The results indicate that both fractions; ethyl acetate and n-butanol are able to inhibit *in vitro* the growth of *A. flavus* and the production

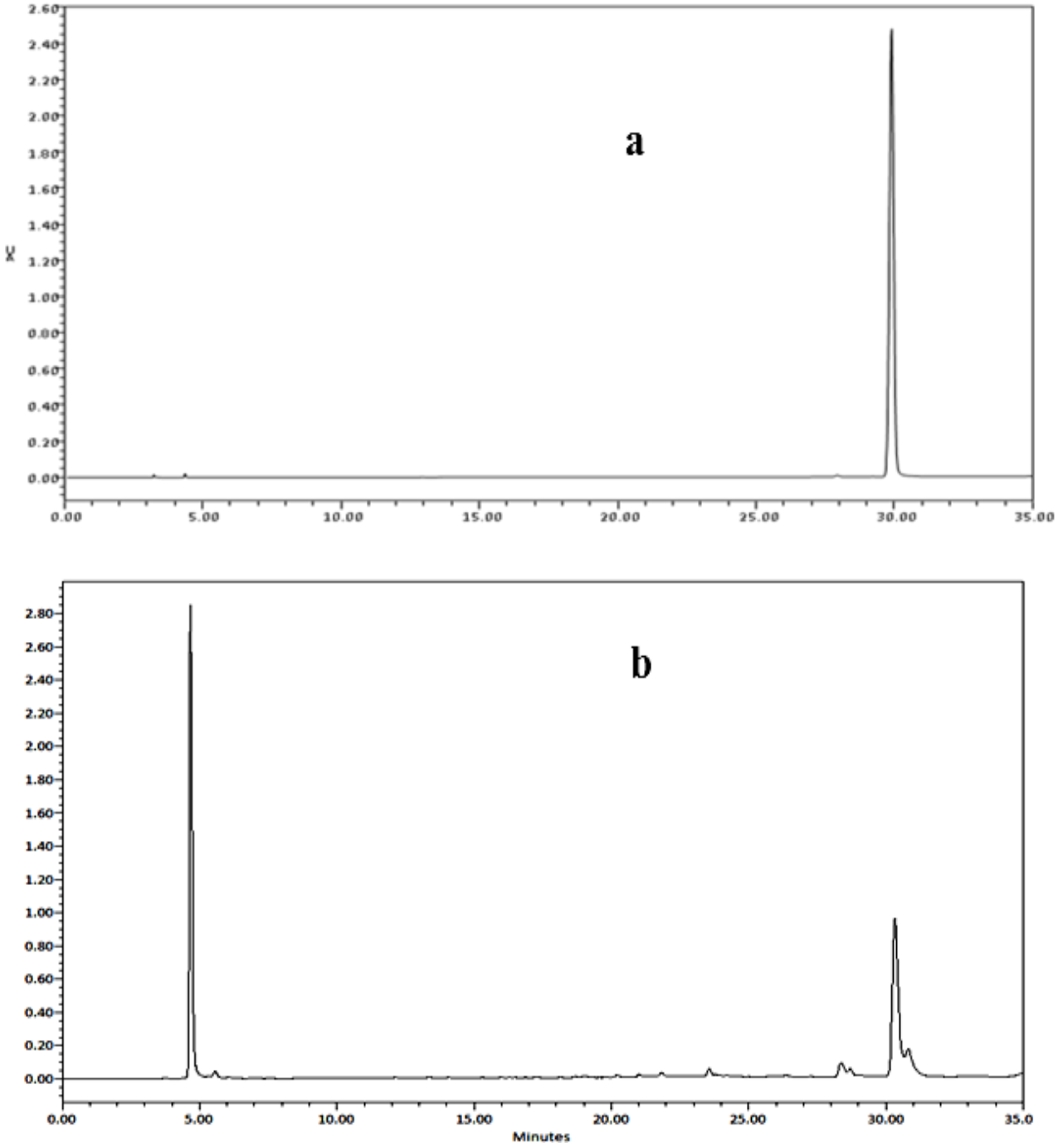

Figure 4. HPLC profile of Aflatoxin B_1 production by *A. flavus*: (a) Aflatoxin B_1 Standard, (b) Aliquot of Mycotoxin of *A. flavus* isolated.

of aflatoxin B1. These fractions must be subjected tofurther study to characterize the active compound, define toxicity and evaluate economic feasibility. The study opens up new opportunities for developing the culture of *L. halimifolium* Mill, firstly to reduce the effect of hepatotoxic and carcinogenic aflatoxins B_1 and secondly to fight against rust peanuts.

CONFLICT OF INTERESTS

The authors declare that they have no conflict of interests.

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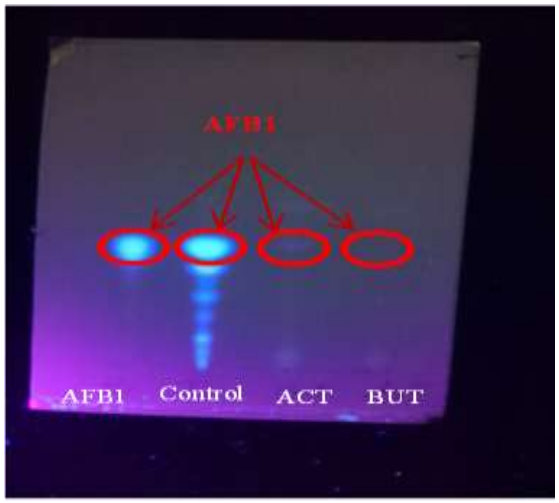

Figure 5. Profile mycotoxins B_1 detection by thin layer chromatography (TLC) of B_1 standard, AMC of control, ethyl acetate and n-butanol fractions of *L. halimifolium* Mill.

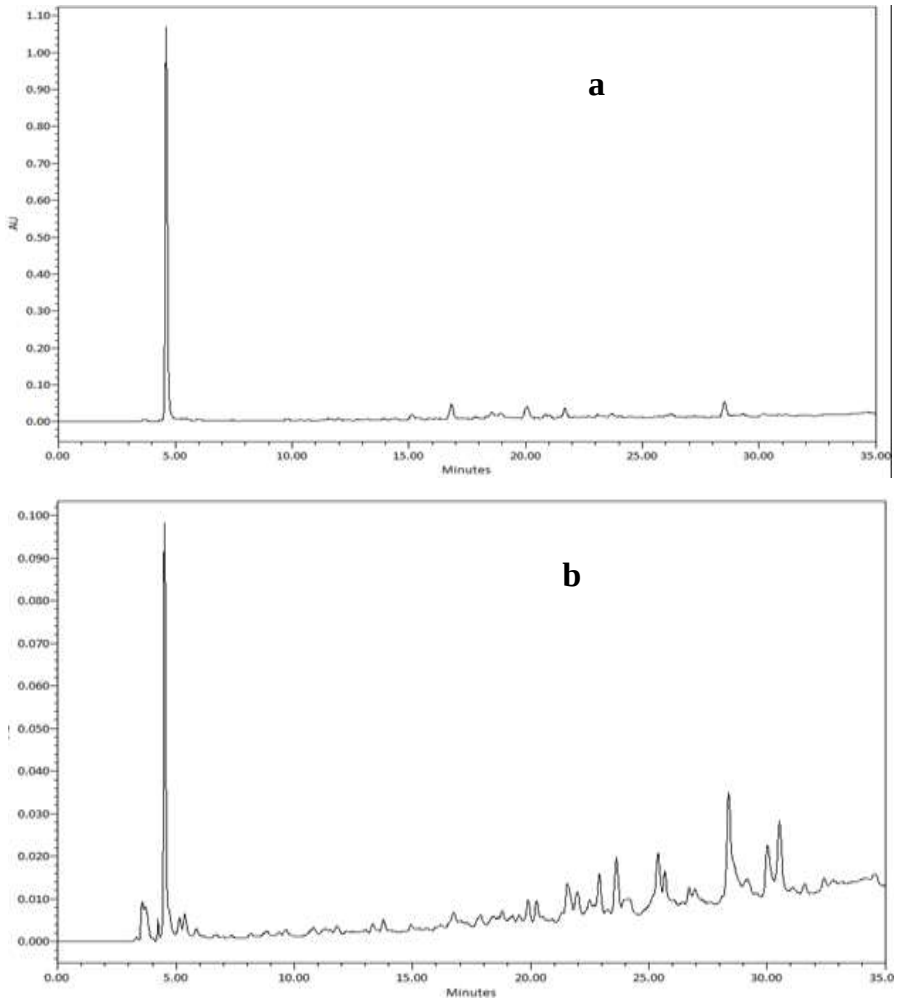

Figure 6. HPLC profile of Aflatoxin B_1 production by *A. flavus*: (c) treated with ACT fraction, (d) treated with BUT fraction of *L. halimifolium* Mill.

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REFERENCES

Abdel Ghani SB, Weaver L, Zidan ZH, Hussein MA, Keevil CW, Brown RCD (2008). Microware-assisted synthesis and antimicrobial activities of flavonoid derivatives. Bioorg. Med. Chem. Lett. 18:518-522.

Alpsoy L 2010. Inhibitory effect of essential oil on aflatoxin activities. Afr. J. Biotechnol. 9(17):2474-2481.

Amagase H, Farnsworth NR (2011). A review of botanical characteristics, phytochemistry, clinical relevance in efficacy and safety of *Lycium barbarum* fruit (Goji). Food Res. Int 44:1702-1717.

Benariba N, Djaziri R, Bellakhdar W, Belkacem N, Kadiata M, Malaisse WJ, Sener A (2013). Phytochemical screening and free radical scavenging activity of Citrullus colocynthis seeds extracts. Asian Pac. J. Trop. Biomed. 3(1):35-40.

Benhammou N, Atik-Bekkara F and Kadifkova-Panovska T (2009). Antioxidant activity of methanolic extracts and some bioactive compound of *Atriplex halimus*. CR Chim. 12(12):1259-1266.

Bluma RV, Etcheverry MG (2008). Application of essential oils in maize grain: impact on *Aspergillus* section *Flavi* growth parameters and aflatoxin accumulation. Food Microbiol. 25(2):324-334.

Botton B, Bertron A, Fevere M, Gauthier S, Guph D, Larpent JP, Reymond P, Sanglier JJ, Vaysser Y, Veau S (1990). Moisissures utiles et nuisibles importance industrielle. Masson collection biotechnologies. Pp. 5-10.

Brown KL, Bren U, Stone MP, Guengerich FP (2009). Inherent Stereo specificity in the Reaction of Aflatoxin B_1 8, 9-Epoxide with Deoxy guanosine and Efficiency of DNA Catalysis. Chem. Res. Toxicol. 22(5):913-917.

Crawford JM, Thomas PM, Scheerer JR, Vagstad AL, Kelleher NL, Townsend CA (2008). Deconstruction of iterative multidomain polyketide synthase function. Science 320:243-246.

Dikbas N, Kotan R, Dadasoglu F, Sahin F (2008). Control of *Aspergillus flavus* with essential oil and methanol extract of *Satureja hortensis*. Int. J. Food Microbiol. 124(2):179-182.

Donno D, Beccaro GL, Mellano MG, Cerutti AK, Bounous G (2015). Goji berry fruit (*Lycium* spp.): Antioxidant compound fingerprint and bioactivity evaluation. J. Funct. Foods 18:1070-1085.

Dutton MF, Ehrlich K, Bennett JW (1985). Biosynthetic relationship among aflatoxins B_1, B_2, M_1 and M_2. Appl. Environ. Microbiol. 49:1392-1395.

Fakruddin Md, Chowdhury A, Nur Hossain Md and Monzur Ahmed M (2015). Characterization of aflatoxin producing *Aspergillus flavus* from food and feed samples. Springer Plus 4:159.

Frisvad JC, Filtenborg O (1983). Classification of *terverticillate Penicillia* based on profile of mycotoxins and other secondary metabolites. Appl. Environ. Microbiol. 46:1301-1310.

Gacem MA, Khelil AO, Gacemi B, Halla N, Djerbaoui An, Bouderhem A, Hadef S, Benreguieg M, Adli DE (2013). Antimycotoxigenic and antifungal activities of Citrullus colocynthis seeds against *Aspergillus flavus* and *Aspergillus ochraceus* contaminating wheat stored. Afr. J. Biotechnol. 12(43):6222-6231.

Ghorbanian M, Razzaghi-Abyaneh M, Allameh A (2008). Study on the effect of neem (*Azadirachta indica A. juss*) leaf extract on the growth of *Aspergillus parasiticus* and production of aflatoxin by it at different incubation times. Mycoses 51:35-39.

Giorni P, Battilani P, Pietri A, Magan N (2008). Effect of awand CO2 level of *Aspergillus flavus* growth and aflatoxin production in high moisture maize post- harvest. Int. J. Food Microbiol. 122:109-113.

Gu S, Wang PL, Jiang R (2007). A study on the preventive effect of *lycium barbarum* polysaccharide on the development of alcoholic fatty liver in rats and its possible mechanism. Chin. J. Hepatol. 3:204-208.

Hermiche R, Daniela B, Dujardin B, Bergman P, Herman F (2012). EFSA's contribution to the implementation of the EU legislation on pesticides residues in food. EFSA J. 10(10):s1011.

Huwig A, Freimund S, Kappeli O, Dutler H (2001). Mycotoxin detoxification of animal feed by different absorbents. Toxicol. Lett. 122:177-188.

Jin M, Huang Q, Zhao K, Shang P (2013). Biological activities and potential health benefit effects of polysaccharides isolated from *Lycium barbarum* L. Int. J. Biol. Macromol. 54:16-23.

Josefa MCJ, Lyibia MC, Sergio MR (2004). Molecular characterization and oenological properties of wine yeasts isolated during spontaneous fermentation of six varieties of grape must. Food Microbiol. 21:149-155.

Martins M, Kluczkovski AM, de Souza TP, Savi CP, Scussel VM (2014). Inhibition of growth and aflatoxin production of *Aspergillus parasiticus* by guaraná (*Paullinia cupanaKunth*) and jucá (*Libidibia ferrea Mart*) extracts. Afr. J. Biotechnol. 13(1):131-137.

McGlynn KA, Hunter K, LeVoyer T, Roush J, Wise P, Michielli RA, Shen FM, Evans AA, London WT, Buetow KH (2003). Susceptibility to aflatoxin B_1-related primary hepatocellular carcinoma in mice and humans. Cancer Res. 63:4594-4601.

Mishra PK, Shukla R, Singh P, Prakash B, Kedia A, Dubey NK (2012). Antifungal, anti-aflatoxigenic, and antioxidant efficacy of *Jamrosa* essential oil for preservation of herbal raw materials. Int. Biodeterior. Biodegradation 74:11-16.

Mohammedi Z, Atik F (2013). Fungitoxic effect of natural extracts on mycelial growth, spore germination and aflatoxin B_1 production of *Aspergillus flavus*. Aust. J. Crop Sci. 7(3):293-298.

Multon JL (1982). Conservation et stockage des grains et graines et produits dérivés : Céréales, oléagineux, aliments pour animaux. Lavoisier Technique & Documentation, Paris Apria. 1, P. 576.

Neveen HA, Deabes M, Lamia AE, Mona K (2015). Chemical Composition and Antifungal Activity of *Ocimum basilicum* L. Essent. Oil Maced. J. Med. Sci. 3(3):374-379.

Outtara-sourabi PB, Nikiema PA, Alfred ST (2011). Caractérisation de souches d'*Aspergillus* spp isolées des graines d'arachides cultivées au Burkina Faso, Afrique de l'Ouest. Int. J. Biol. Chem. Sci. 5(3):1232-1249.

Pamel EV, Vlaemynck G, Heyndrickx M, Herman L, Verbeken A, Daeseleire E (2011). Mycotoxin production by pure fungal isolates analysed by means of an uhplc-ms/ms multi-mycotoxin method with possible pitfalls and solutions for patulin-producing isolates. Mycotoxin Res. 27(1):37-47.

Paranagama PA, Abeysekera KHT, Abeywickrama KP and Nugaliyadde L (2003). Fungicidal and anti-aflatoxigenic effects of the essential oil of Cymbopogon citrates (DC) staps. (lemongrass) against *Aspergillus flavus* link isolated from stored rice. Lett. Appl. Microbiol. 37:86-90.

Pitt JI, Hocking AD (2009). Fungi and Food spoilage, 2nd edn. Blackie Academic and Professional, London.

Potterat O (2010). Goji (*Lycium barbarum* and *L. chinense*): Phytochemistry, pharmacology and safety in the perspective of traditional uses and recent popularity. Planta Med. 76(1):7-19.

Pusztahelyi T, Holb IJ, Pócsi I (2015). Secondary metabolites in fungus-plant interactions. Front. Plant Sci. 6:573.

Rasooli I, Fakoor MH, Yadegarinia D, Gachkar L, Allameh A, Rezaei MB (2008). Antimycotoxigenic characteristics of *Rosmarinus officinalis* and *Trachyspermum copticum* L. essential oils. Int. J. Food Microbiol. 122(1-2):135-139.

Reddy KRN, Salleh B, Saad B, Abbas HK, Abel CA, Shier WT (2010). An overview of mycotoxin contamination in foods and its implications for human health. Toxin Rev. 29:3-26.

Stephen IB, Lua H, Kaoa TH, Chena H (2010). Simultaneous determination of phenolics acids and flavonoids in *lycium barbarium* linnaeous HPLC-DAD-ESI-SM. J. Pharm. Biomed. Anal. 51:549-556.

Streit E, Schatzmayr G, Tassis P, Tzika E, Marin D, Taranu I, Tabuc C, Nicolau A, Aprodu I, Puel O, Oswald IP (2012). Current Situation of Mycotoxin Contamination and Co-occurrence in Animal Feed-Focus on Europe. Toxins 4:788-809.

Trail F, Mahanti N, Linz J (1995). Molecular biology of aflatoxin biosynthesis. Microbiology 141:755-765.

Wagacha JM, Muthomi JW (2008). Mycotoxin problem in Africa: current status, implications to food safety and health and possible management strategies. Int. J. Food Microbiol. 124(1):1-12.

Xianwen Lai, Zhang H, Liu R, Liu C (2015). Potential for aflatoxin B_1 and B_2 production by *Aspergillus flavus* strains isolated from rice

sample. Saudi J. Biol. Sci. 22(2):176-180.
Xiaq J, Liong AC, Ching YP, Chan GRC, So KF, Fung ML, Tipoe GL (2012). *Lycium barbarum* polysaccharides, protect mice liver from carbon tetrachloride-induced oxidative stress and nectoinflammation. J. Ethnopharmacol. 139:462-470.


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