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Heat stable antimicrobial activity of *Burkholderia gladioli* OR1 against clinical drug resistant isolates

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***Background & objectives*: Drug resistant microbes are a serious challenge to human health. During the search for novel antibiotics/inhibitors from the agricultural soil, a bacterial colony was found to inhibit the growth of clinical isolates including *Staphylococcus* (resistant to amikacin, ciprofloxacin, clindamycin, clinafloxacin, erythromycin, gentamicin and methicillin) and *Candida* (resistant to fluconazole and itraconazole). The culture was identified as *Burkholderia gladioli* and produced at least five different antimicrobial compounds which were highly stable at high temperature (121°C) and in the broad *p*H range (3.0-11.0). We report here the antimicrobial activity of *B. gladioli* against drug resistant bacterial pathogens.**

***Methods*: The bacterial culture was identified using morphological, biochemical and 16S rRNA gene sequencing techniques. The antimicrobial activity of the identified organism against a range of microbial pathogens was checked by Kirby-Bauer's disc diffusion method. The antimicrobial compounds in the cell free supernatant were chloroform-extracted and separated by thin layer chromatography (TLC).**

***Results*: *B. gladioli* OR1 exhibited broad spectrum antimicrobial activity against drug resistant clinical isolates belonging to various genera of bacteria (*Staphylococcus, Enterobacter, Enterococcus, Acinetobacter* and *Citrobacter*) and a fungus (*Candida*). Based on TLC profile and bioautography studies, the chloroform extract of *B. gladioli* OR1 consisted of at least three anti-staphylococcal and two anti-*Candida* metabolites. The antimicrobial activity was heat stable (121°C/20 min) as well as *p*H stable (3.0-11.0).**

***Interpretation & conclusions*: The bacterial soil isolate, *B. gladioli* OR1 possessed the ability to kill various drug resistant bacteria and a fungus. This organism produced many antimicrobial metabolites which might have the potential to be used as antibiotics in future.**

Key words Antimicrobial activity - *Burkholderia gladioli* OR1 - *Candida* - chloroform extract - *Staphylococcus*

Staphylococcus and *Candida* are the two major agents causing wide array of microbial diseases, especially in infants and immuno-compromized hosts[1,2]. If left untreated, these diseases can be fatal[3,4]. Although many antibiotics are available for the treatment of staphylococcal and *Candida*

infections, the choice is becoming limited because of the emergence of antibiotic resistance in the clinical isolates belonging to these genera[5,6]. The isolation of multi-drug resistant *Staphylococcus* and *Candida* is a frequent observation[7,8]. Looking at the alarming situation created by the development of drug resistant pathogens, a concerted effort is being made by the scientists to search for new antimicrobial compounds that can kill or inhibit the growth of such drug resistant microbes.

Antibiotic production is generally considered to be the domain of fungi and actinomycetes. However, a few bacteria have also been reported to produce antibiotics[9,10]. During the screening for novel bio-active compounds of microbial origin, a bacterial colony, isolated from rhizospheric soil, subsequently identified as *Burkholderia gladioli* OR1 exhibited inhibitory properties. Here, we report the antimicrobial activity of *B. gladioli* against clinical drug resistant bacterial and fungal pathogens of human origin.

Material & Methods

Culture isolation and identification: This study was conducted in the Department of Biotechnology, Panjab University, Chandigarh, India. A spoonful of agricultural soil, obtained from a village Khuda Lahora, near Chandigarh (India), was suspended in 20 ml sterile distilled water, thoroughly agitated and allowed to stand at room temperature for 10 min. Ten-fold dilutions of the aqueous phase of the soil mixture were made, spread-plated on nutrient agar (NA) plates and incubated at 30°C for 48 h. One bacterial colony found to inhibit the growth of adjoining microbial colonies, was streaked on a fresh NA plate, incubated at 30°C for 24 h and stored at 4°C. The isolated bacterial culture was identified based on morphological[11], biochemical[12] and 16S rRNA gene sequencing data[13].

Extraction of antimicrobial compounds from B. gladioli OR1: *B. gladioli* OR1 (1.0% inoculum) was grown in 100 ml of glucose yeast extract (GYE, glucose- 1.0%, yeast extract- 0.05%; *p*H- 7.0) broth contained in a 500 ml conical flask, under standard conditions *i.e.*, at 30°C, 130-150 rpm in an orbital shaker (Shaker incubator - New Brunswick Scientific Co., Inc., Edison, USA) for 24 h. The culture was centrifuged (10,000 *g*, 10 min, 4°C) (High speed refrigerated centrifuge - Hitachi, Japan), and the cell free culture supernatant was mixed and agitated with equal volume of distilled chloroform (Thermo Fischer Scientific, USA) in a separating funnel (Borosil Glassworks, India). The chloroform layer was separated and concentrated on a rotary vacuum evaporator (35°C /100 rpm, BUCHI, Switzerland). The solid brownish mass, left after the evaporation, was dissolved in chloroform in a concentration of 20 mg/ml and stored at -20°C for further use.

Antimicrobial activity of B. gladioli OR1 against clinical isolates: The antimicrobial activity (AMA) of the chloroform extract of *B. gladioli* OR1 (CEBG) against clinical isolates of bacteria and fungi (obtained from Department of Microbiology, Government Medical College and Hospital, Chandigarh) was determined by Kirby-Bauer's disc-diffusion method[14]. The clinical isolates included 20 isolates of *Staphylococcus aureus* obtained from various sources like pus, semen, tissue cells, tracheal tip, umbilical tip and wound; 14 isolates of *Pseudomonas aeruginosa* from blood, pus, trachea, urine and wound; 11 of *Acinetobacter* CBC isolated from ascites fluid tap, blood, pus, sputum, trachea, tracheotomy pus, urine and wound; 11 *Escherichia coli* isolates from catheter tip, pleural fluid, pus, sputum, trachea and urine; 6 *Klebsiella pneumoniae* isolates from urine and wound; 3 *Citrobacter freundii* isolates from urine; 3 *Enterobacter* species isolates from ascites fluid tap and blood; 1 *Enterococcus* species isolate from blood; 4 isolates each of *Candida albicans* and *C. tropicalis* from blood and urine. One hundred microlitres of ten-fold dilutions of actively growing (mid log phase) bacterial (both Gram-positive & Gram-negative) and yeast cultures (clinical isolates) having 0.5 McFarland turbidity were spread-plated uniformly (using a sterile glass spreader) onto NA and yeast extract peptone dextrose agar (YEPDA) (Hi-Media, Mumbai, India) plates, respectively. These plates were then allowed to dry in the laminar flow chamber for 5 min. Ten microlitres of the chloroform extract (conc.- 20 mg/ml) of *B. gladioli* OR1, was gently transferred to the sterile filter paper disks (6 mm diameter, Hi-Media, India) which were air dried for two min and then placed carefully onto cultured NA/YEPDA plates along with the pure chloroform-treated, dried disks as control. The plates were incubated for 24 h at 30°C (for yeast) and 37°C (for bacteria). The AMA was determined by measuring the diameter of the zone of inhibition of bacterial and fungal growth around the discs.

Association between B. gladioli OR1 growth and antimicrobial activity in GYE broth: A total of six flasks containing GYE broth inoculated with 1.0 per cent overnight grown *B. gladioli* OR1 culture, were incubated for different time intervals (24, 48 and 72 h), in duplicates under shaking conditions (130 rpm)

at 30°C. After each time interval, two flasks were withdrawn and the absorbance of the cultures measured at 600 nm using a UV/VIS spectrophotometer (Lab India UV 3000+, India). The *B. gladioli* OR1 cultures were then centrifuged, the cell free supernatant pooled and the chloroform extract of the cell free supernatant was prepared and concentrated. The AMA of the chloroform extracts, obtained after each time interval were checked by Kirby-Bauer's disc-diffusion method using test microorganisms *S. aureus* (resistant to amikacin, ciprofloxacin, clindamycin, clinafloxacin, erythromycin, gentamicin and methicillin) and *C. tropicalis* (resistant to fluconazole and itraconazole).

Thin layer chromatography- Bioautography: The AMA of the chloroform soluble fraction of the supernatant of *B. gladioli* OR1 culture was qualitatively analyzed using Gibbons and Gray[15] TLC bioautography overlay assay. CEBG (20 µl) was spotted onto pre-coated silica gel 60 TLC strips (F 254, Merck Co, USA). The strips were developed in a solvent system [chloroform: methanol (9: 1)], air dried, examined under UV light (254 & 366 nm) and carefully laid over the solid medium (NA or YEPDA) plates. Finally, 20 ml of the soft agar media (agar conc.- 1.0%) containing 100 µl of actively growing cells ($N=10^8$ cells/ml) either of multi-drug resistant *S. aureus* or *C. tropicalis*, was gently laid over the developed TLC strips, placed on the surface of the respective medium plates, and incubated at 30°C (for *C. tropicalis*) or 37°C (for *S. aureus*) for 24 h. The R_f values of the antimicrobial zones exhibited by the compounds, separated on the TLC strips were calculated.

Stability of antimicrobial compounds: The stability of antimicrobial compounds (AMCs) present in the cell free culture supernatant of *B. gladioli* OR1 [filtered through 0.22 µm PTFE membrane filter, 25 mm (Hangzhou Anow Microfiltration Co., Ltd., China)], towards heat and *p*H was determined.

Heat stability: Ten millilitres each of the cell free culture supernatant of *B. gladioli* OR1 were dispensed in various 50 ml screw capped conical flasks. The flasks were subjected to heat treatment (50, 70, 100 & 121°C) for 20 min either in a water bath (up to 100°C) or in an autoclave (121°C).

pH stability: Ten millilitres each of cell free culture supernatant contained in different flasks were individually adjusted to various *p*H values (3.0, 5.0, 9.0, 11.0) using 0.1 M HCl or 0.1 M NaOH. Same amount of sterile distilled water was added to control samples. The samples were then kept at room temperature for one hour, chloroform extracted and assayed for AMA against multi-drug resistant *S. aureus* and *C. tropicalis*.

Results & Discussion

In the genus *Burkholderia*, mainly *B. cepacia* has been well documented to possess antimicrobial properties and some of these compounds have been characterized[16-20]. However, there are only a few reports on the antimicrobial nature of other species of *Burkholderia* including *B. gladioli*. Although a few studies have reported the antimicrobial activity of *B. gladioli* against plant pathogens belonging to the genera *Alternaria*, *Aspergillus*, *Penicillium*, *Clavibacter*, *Listeria*, *Acidovorae*, *Acetobacter*, *Monilia*, *Botrytis* and *Rhodotorula*[21-23], there is no report on the AMA of *B. gladioli* against clinical drug resistant pathogens of human origin. This observation, as well as the emergence of drug resistant microbial pathogens to currently used antibiotics led us to explore the antimicrobial profile of our isolate *B. gladioli* OR1 against clinical strains.

Microbial pathogens belonging to the genera *Staphylococcus* and *Candida* are posing serious health hazard, because of the isolation of multi-drug resistant strains from hospitals all over the world[24-26]. In the preliminary experiments (data not shown), a clear zone of inhibition around the *B. gladioli* OR1 colony patched in the centre of uniformly pre-streaked multi-drug resistant *S. aureus* and *C. tropicalis* plates indicated the ability of this organism to inhibit these drug resistant microbial isolates. Subsequent experiments proved that AMA was extracellular in nature and was chloroform soluble (data not shown). The chloroform extract of the cell free supernatant of *B. gladioli* OR1 was found to inhibit the growth of all the 20 clinical isolates of *S. aureus* tested. Of these 20, 10 isolates were sensitive to many antibiotics namely amikacin, amoxicillin-clavulanic acid, ciprofloxacin, clindamycin, clinafloxacin, erythromycin, gentamicin, linezolid, methicillin and vancomycin. The remaining 10 isolates of *S. aureus* were resistant to one or more antibiotics; four were resistant to a single antibiotic (clinafloxacin or gentamicin), two were resistant to two antibiotics (erythromycin and gentamicin), and four were multi-antibiotic resistant (amikacin, clinafloxacin and gentamicin; amikacin, ciprofloxacin, erythromycin and gentamicin; amikacin, ciprofloxacin, clindamycin, clinafloxacin, erythromycin, gentamicin and methicillin). The antimicrobial property of CEBG was confined not only to Staphylococci, but also to

other bacterial pathogens namely, *Acinetobacter*, *Enterococcus*, *Enterobacter* and *Citrobacter freundii* (Table) isolated from various sites of human patients. However, CEBG did not inhibit the growth of *E. coli*, *K. pneumoniae* and *P. aeruginosa*. These results suggested that CEBG had a wide range of AMA against Gram positives but selective AMA against Gram negatives.

Similarly, various species of drug resistant isolates of *Candida*, including *C. albicans* and *C. tropicalis* were inhibited by CEBG. Of the four isolates of *C. tropicalis* tested, one was sensitive to amphotericin B, fluconazole and itraconazole; one resistant to a single anti-fungal agent fluconazole and two were resistant to fluconazole and itraconazole. Of the three isolates of *C. albicans* inhibited by CEBG, one was sensitive to amphotericin B, fluconazole and itraconazole; two were resistant to single anti-fungal agent, either amphotericin B or fluconazole.

An association between *B. gladioli* OR1 growth and AMA was observed in GYE medium (Fig. 1). Although highest OD of 3.16 was observed after 48 h of incubation, but maximum AMA was observed during 'mid log phase' (OD=2.20) *i.e.*, after 24 h of incubation and remained almost same thereafter, suggesting that very actively growing culture possessed maximal AMA. This observation was interesting because majority of microbes produce antibiotics (penicillin, streptomycin, gentamicin, cephalosporin, *etc*.) in their

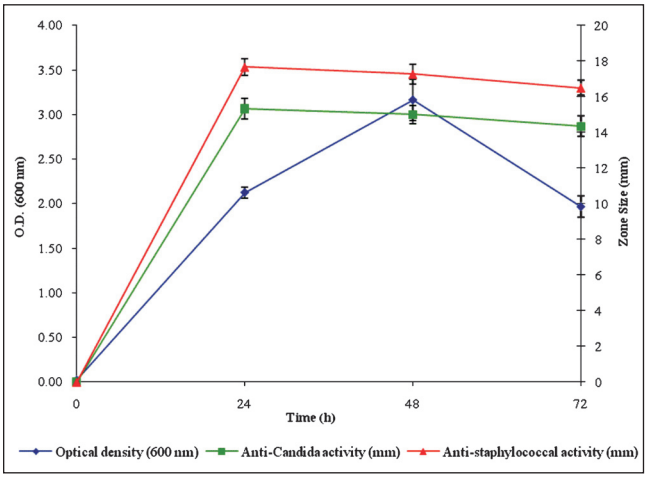

Fig. 1. Growth and antimicrobial activity of *B. gladioli* OR1 (Growth conditions: GYE broth, *p*H-7.0, 30°C, 130 rpm, 1% inoculum. Values are mean ± SD of three individual experiments).

Table. Anti-microbial activity of *B. gladioli* OR1 against clinical isolates

S. No.	Test organisms (no. of strains)	Antibiotic resistance	Zone of inhibition of microbial growth (diameter in mm)	Mean zone of inhibition of microbial growth (diameter in mm)
1.	*Acinetobacter* CBC (7)	AMC, AMK, ATM, CAZ, CIP, CLA, CRO, CTX, CXM, GEN, NIT, PIP, TZP, ZOX	8.0-12.0	10.0
2.	*Citrobacter freundii* (2)	AMK, AMP, CAZ, CIP, CRO, CTX, CXM	9.0	9.0
3.	*Enterobacter* sp. (2)	AMC, AMK, CAZ, CXM, FEP, GEN, ZOX	45.0	45.0
4.	*Enterococcus* sp. (1)	AMC, AMK, ERY, GEN, LEX	20.0	20.0
5.	*Staphylococcus aureus* (10)	AMK, CIP, CLI, CLX, ERY, GEN, MET	15.0-33.0	24.0
6.	*S. aureus* (10)	Sensitive to all antibiotics tested*	10.0-24.0	17.0
7.	*Candida albicans* (2)	AMB, FLC	11.0-11.5	11.25
8.	*C. albicans* (1)	Sensitive to all antibiotics tested**	12.0	12.0
9.	*C. tropicalis* (3)	FLC, ITC	12.0-13.5	12.75
10.	*C. tropicalis* (1)	Sensitive to all antibiotics tested**	13.0	13.0

The control sterile filter paper disc, loaded with 10 µl of chloroform and dried for 2 min at room température, did not exhibit any antimicrobial activity.

*Antibiotics tested - AMK, AMC, AMP, ATM, FEP, CTX, CAZ, ZOX, CRO, CXM, LEX, CIP, CLA, CLX, CLI, ERY, GEN, MET, NIT, PIP & TZP; **Antibiotics tested - AMB, FLC & ITC

AMK, amikacin; AMC, amoxicillin-clavulanic acid; AMB, amphotericin B; AMP, ampicillin; ATM, aztreonam; FEP, cefepime; CTX, cefotaxime; CAZ, ceftazidime; ZOX, ceftizoxime; CRO, ceftriaxone; CXM, cefuroxime; LEX, cephalexin; CIP, ciprofloxacin; CLA, clavulanic acid; CLX, clinafloxacin; CLI, clindamycin; ERY, erythromycin; FLC, fluconazole; GEN, gentamicin; ITC, itraconazole; MET, methicillin; NIT, nitrofurantoin; PIP, piperacillin; TZP, piperacillin-tazobactam

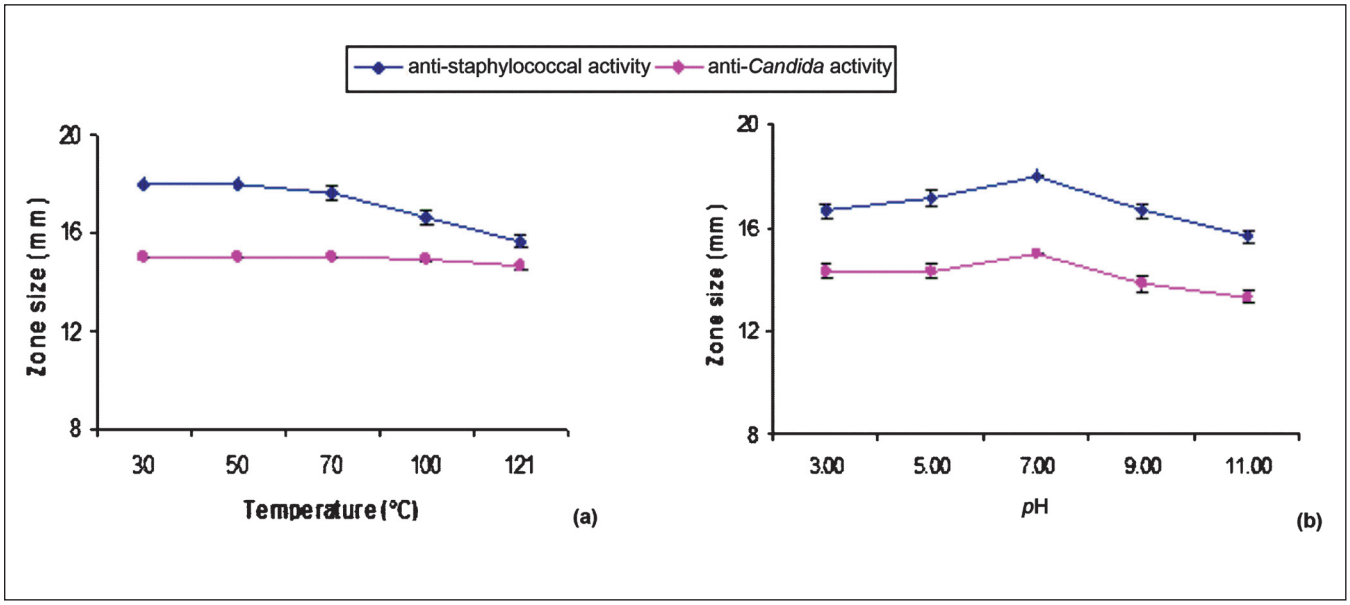

Fig. 2. Effect of temperature **(a)** and *p*H **(b)** on antimicrobial activity of the cell free supernatant of *B. gladioli* OR1. Values are mean ± SD of three individual experiments.

stationary phase rather than log phase of growth. Some bacteriocins[27] and an antibiotic, pyrrolnitrin[28] have also been earlier reported to be produced in the log phase.

The AMA of the cell free culture supernatant of *B. gladioli* OR1 was found to be highly heat stable as well as *p*H stable (Fig. 2). The anti-*Candida* activity was 100 per cent stable when the sample was heated up to 100°C for 20 min but a small decrease of 1.4 per cent occurred in the autoclaved (121°C for 20 min) sample. The anti-staphylococcal activity was 100 per cent heat stable up to 50ºC, however, a decrease of 2.8, 8.4 and 13.9 per cent was observed when the samples were heated at 70, 100 and 121°C, respectively. The production of heat stable antimicrobial compounds in the log phase of bacterial growth is not uncommon; some of the bacteriocins belonging to class II have been reported to be heat stable[27]. The AMA was quite stable in the *p*H range 3.0-11.0, with maximum stability observed at *p*H 7.0. Only a slight decrease in AMA was observed when the *p*H of the sample was adjusted to the acidic or alkaline values *i.e.*, at 3.0, 5.0, 9.0 and 11.0, the sample retained 86.1-94.4 per cent of the anti-staphylococcal activity and 90.0-96.6 per cent of the anti-*Candida* activity. The *p*H and temperature data indicate that *B. gladioli* OR1 produces more than one AMC.

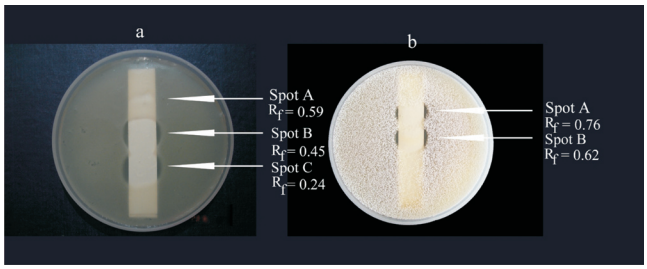

Fig. 3. Bioautography exhibiting anti-*S. aureus* **(a)** and anti-*C. tropicalis* **(b)** activities of *B. gladioli* OR1.

The CEBG containing AMCs was subjected to silica gel TLC analysis in a solvent system comprising of chloroform: methanol:: 9: 1. The analysis of the separated compounds under UV light (254 and 365 nm) [and upon derivatizing the TLC with methanolic-sulphuric acid (10.0%)], revealed at least five major spots with R_f values 0.24, 0.45, 0.59, 0.62 and 0.76. In order to find out the correlation of these spots with antimicrobial property, bioautography of the freshly developed air-dried TLC strips containing separated components of the extract was performed, using multi-drug resistant *S. aureus* and *C. tropicalis* as target organisms. The results (Fig. 3) exhibited that *B. gladioli* OR1 produced at least three anti-staphylococcal compounds (R_f values: 0.24, 0.45 & 0.59) and two anti-*Candida* compounds (R_f values: 0.62 & 0.76).

The present study emphasizes the importance of *B. gladioli* for the production of various AMCs which possess the potential to kill drug resistant pathogenic

microbes. Once the compounds are purified and characterized, these can act as lead molecules in the design of novel microbial inhibitors.

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