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PURIFICATION AND STRUCTURAL CHARACTERIZATION OF A PHTHALATE ANTIBIOTIC FROM *BURKHOLDERIA GLADIOLI* OR1 EFFECTIVE AGAINST MULTI-DRUG RESISTANT *STAPHYLOCOCCUS AUREUS*

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ABSTRACT

A soil isolate, identified as *Burkholderia gladioli* OR1, has been reported in our earlier study, to possess broad spectrum antimicrobial activity against drug resistant clinical isolates of bacteria and fungi. The 16S rRNA gene sequence data of *B. gladioli* strain OR1 has been deposited in the GenBank databases (MTCC) under the accession number MTCC11251. Thin layer chromatography and bioautography studies of chloroform extract of the cell free supernatant of this organism, showed at least four different antimicrobial compounds. The silica gel column chromatography and high performance liquid chromatography led to the isolation of one compound that exhibited growth inhibitory activity against multi-antibiotic resistant clinical isolates of *Staphylococcus aureus* (resistant to amikacin, ciprofloxacin, clindamycin, clinafloxacin, erythromycin, gentamicin and methicillin). On the basis of mass, infra-red (IR), ^{1}H nuclear magnetic resonance (NMR), ^{13}C NMR and 2D NMR spectral studies, we propose the structure of this antibiotic to be bis-(2-ethylhexyl) phthalate, isolated for the first time from the species of the genus *Burkholderia*.

Keywords: Antibiotic resistant, chloroform extract, chromatography, silica gel, spectroscopy

INTRODUCTION

The number and complexity of infections caused by microbial pathogens are continuously rising and have become a real problem today. To worsen the situation further, antimicrobial resistance among clinical microbial pathogens has been increasing steadily during the past several decades, leading to the isolation of multi-drug resistant pathogens from hospitals all over the world. *Staphylococcus aureus* is one of the most important pathogens posing serious health hazard, because of its exceptional virulence, stress tolerance and high capacity to induce antimicrobial resistance (**De Leo and Chambers, 2009**).
In the absence of any effective vaccine against *S. aureus*, it is imperative that new antimicrobials are identified to combat infections mediated by drug-resistant *S. aureus*. Natural products derived from "microbes" have long been an inspiration for the development of drugs with applications in the field of human health (**Stewart *et al.*, 2004**). In the incessant search for less toxic, more potent antibiotics responsive to the major pathogens which have acquired resistance to the existing antibiotics, a novel strain *Burkholderia gladioli* OR1, isolated from rhizospheric soil, was found to exhibit activity against multi-drug resistant (MDR) clinical pathogen, *S. aureus*. Based on the thin layer chromatography (TLC) and bio-autography studies, it produced two heat stable (100°C/20 min) (373.15 K/1200 s) and chloroform soluble antimicrobial compounds (**Bharti *et al.*, 2011**). The objective of our study was the purification and characterization of atleast one of these active compounds.

MATERIALS AND METHODS

Production and preparation of crude extract

The nutrient medium and incubation conditions required for the growth of *B. gladioli* OR1, as well as the preparation of chloroform extract (possessing anti-staphylococcal activity) from cell free culture supernatant have been mentioned in our earlier publication (**Bharti *et al.*, 2011**). In brief, *B. gladioli* OR1 was cultivated in glucose yeast extract medium (glucose- 1.0% w/v, yeast extract- 0.05% w/v; pH- 7.0) at 30°C for 24 h (303.15 K for 86400 s) under shaking conditions. The culture was centrifuged (HITACHI, Japan) and the cell free supernatant was extracted with equal volume of chloroform. The organic fraction was separated and concentrated in a vacuum rotavapour (35°C/100 rpm, BUCHI, Switzerland) yielding brownish paste, which was tested for activity against clinical pathogens including MDR *S. aureus* (obtained from Department of Microbiology, Government Medical College and Hospital, Chandigarh, India).

Purification of the antimicrobial compound

Initial fractionation of the brownish paste containing antimicrobial compounds, was done using vacuum liquid chromatography (VLC), in which 22.0 g (0.022 kg) of the crude chloroform extract of *B. gladioli* OR1 was dry loaded gently over the silica gel G (CDH Fine-chem., India) bed made into a column of 10 inch diameter X 14 inch height (0.254 m X 0.355 m) in a G-4 sintered glass funnel. Elution was done under vacuum, using a step-wise gradient of solvents, starting with 100% hexane, then adding hexane-ethyl acetate increasing ethyl acetate up to 100% and finally giving a wash with methanol : ethyl acetate (1 : 1). A total of 50 fractions (250 ml i.e., 0.0003 m^3 each) were collected and pooled on the basis of TLC profiling to give sixteen major pools. The active fraction (exhibiting activity against MDR *S. aureus*) obtained from VLC using 5% (v/v) methanol in ethyl acetate, exhibited minimum number of separable spots, as determined by TLC-bioautography and was chosen for further purification using flash column chromatography (FCC). In FCC, a column (dimensions - 18 cm X 1.5 cm i.e., 0.18 m X 0.015 m) was packed with 12 g (0.012 kg) activated (heating for 1 h at 110°C or 3600 s at 383.15 K) silica gel (230-400 mesh size) and equilibrated using chloroform. The isocratic elution of the loaded active fraction was done using $CHCl_3$: CH_3OH (chloroform-methanol) :: 95 : 5 (v/v) yielding 65 fractions (25 ml or 2.5 X 10^{-5} m^3 each), which were pooled into 5 major fractions based on the TLC analysis.
The fractions eluted from FCC, whose RP-TLC (reverse phase TLC- MERCK Co., USA) revealed one major spot were then subjected to analytical high performance liquid chromatography (HPLC) {SHIMADZU-SCL-10AVP (Japan), injection volume - 10 µl of 1 mg ml^{-1} (1 µl = 10^{-9} m^3, 1 mg = 10^{-6} kg) sample, column - Phenomenex C_{18} 250 mm X 4.6 mm (0.025 m X 0.0046 m), flow rate - 1 ml min^{-1}, detection wavelength - 252 nm, elution gradient - 0-30 min – 50-100% v/v methanol in water}. The fraction showing a few separable peaks in the max plot from analytical HPLC was further purified using semi preparative HPLC {SHIMADZU-SCL-10AVP, injection volume - 100 µl of 30 mg ml^{-1} filtered sample in the SIL-20AC auto-sampler, column - RP-Princeton 250 mm X 20 mm (0.25 m X 0.02 m), flow rate - 3 ml min^{-1}, solvent system - 58% v/v methanol (HPLC Grade- J.T. BAKER, USA) in water isocratic}.

Antimicrobial assay of the isolated compound

The pure compound obtained was checked for antagonistic activity using the same assays as done earlier for the crude extract i.e., disc-diffusion method and TLC-bioautography technique (**Bharti *et al*., 2011**) against clinical drug resistant *S. aureus* strains. In brief, for disc-diffusion assay, ten microlitres (10^{-8} m^3) of the purified sample obtained from semi-preparative HPLC, was applied on each sterile filter paper discs (6 mm diameter, HI-MEDIA, India) (0.05 mg ml^{-1} of methanol i.e., 0.5 µg/disc). The impregnated discs were air dried and placed on each nutrient agar plate, spreaded individually with 10 strains of antibiotic resistant *S. aureus* isolated from various sites of human patients (Tab 1). For bioautography assay, 20 µl (2 X 10^{-8} m^3) of this pure compound was applied on silica gel 60 TLC strip (F 254, MERCK Co, USA), developed in $CHCl_3$: CH_3OH :: 9 : 1 (v/v), air-dried and examined under ultra violet (UV) light (254 nm & 366 nm). This TLC strip was then placed on a media plate which was then overlaid with soft nutrient agar (agar- 1.0% w/v) containing actively growing MDR *S. aureus*. The plates for both the assays were incubated at 30ºC for 24 h and the zone diameters and R_f value calculated respectively.

Structural characterization of the purified antimicrobial compound

Once pure active compound was isolated, its identity was determined by spectroscopic methods including mass, IR and NMR spectral studies. The molecular mass of the isolated compound was ascertained by MS instrument (WATERS Q-ToF Micro, USA) in a suitable solvent (methanol) over a range of 0-1000 *m/z* in API-ES-MS, by injecting at a continuous flow of 5 µl min^{-1} with set ionization parameters (capillary dissolvation voltage and temperature: 3000 V & 200ºC). IR spectrum of the compound was taken in solubilized form on a KBr window to get an idea about the various functional groups present, using FT-IR (fourier transform IR-PERKIN ELMER, USA). The active pure compound was then submitted for ^1H (400 MHz), ^{13}C (100 MHz) and 2-Dimensional NMR spectral studies including distortionless enhancement by polarization transfer spectroscopy (DEPT), ^1H-^1H homonuclear correlated spectroscopy (^1H-^1H-COSY), ^1H-^{13}C heteronuclear multiple bond coherence spectroscopy (^1H-^{13}C-HMBC) and ^1H-^{13}C heteronuclear multiple quantum coherence spectroscopy (^1H-^{13}C-HMQC) (AVANCE III 400 BRUCKER, Switzerland) using TMS (tetra methyl-silane) as the internal standard.

RESULTS

The cell free culture supernatant of *B. gladioli* OR1 was extracted with chloroform, which was then concentrated to yield a solid brownish mass (yield-200 mg per litre). This dry residue was stored at -20°C for further use.

Fractionation of the crude extract

Out of the 16 major fractions obtained from VLC of the chloroform extract of *B. gladioli* OR1, fractions 7^{th} to 16^{th}, eluted using comparatively polar solvent (50% - 100% v/v ethyl-acetate in hexane and upto 50% v/v methanol in ethyl acetate) were found to be active and exhibited at least a single zone against MDR *S. aureus* upon bioautography. Out of these, fraction 12^{th} (200 mg) exhibited separable spots on TLC (under UV light), and hence was further chosen for purification using FCC. Out of the 5 major fractions of 12^{th} i.e., 12-1-F to 12-5-F, 12-2-F exhibited single major spot on RP-TLC.

Isolation of pure antimicrobial compound

Analytical HPLC of 12-2-F (30 mg or 3X10^{-5} kg) exhibited 4 major peaks at different retention times methanol-water (CH_3OH-H_2O) gradient. Using semi-preparative HPLC, a total of 5 peaks of 12-2-F were thus collected (12-2F-1 to 12-2F-5) at various retention times using a solvent mixture CH_3OH-H_2O.
Profiling of the light brown fraction 12-2F-4 (4 mg) using analytical HPLC revealed a single peak in the chromatogram thus confirming its purity (Fig. 1).

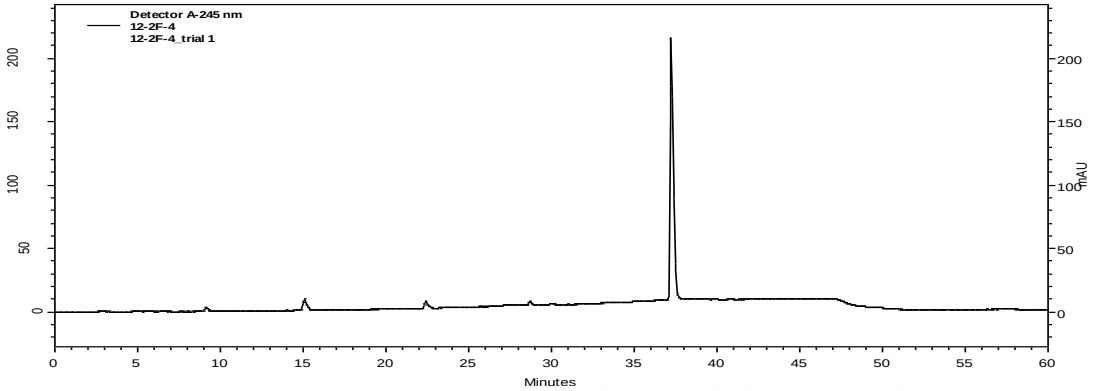


Figure 1 Analytical HPLC chromatogram depicting single peak in 12-2F-4 fraction purified from the extract of *Burkholderia gladioli* OR1.

Anti-*Staphylococcus aureus* activity of the pure compound

The pure compound 12-2F-4 obtained, was found to exhibit appreciable anti-*S. aureus* zone of inhibition (13.5-14 mm i.e., 0.0135-0.014 m diameter) (Fig. 2) against a number of antibiotic resistant clinical strains (Tab 1).

Three major anti-*S. aureus* bands were obtained (Fig. 3A). 12-2F-4 exhibited a single TLC band when visualized under UV light (254 nm) (Fig. 3B), further emphasizing its pure nature. This band at R_f value of 0.45 was one of the major bands present in the crude extract of *B. gladioli* OR1 (Fig. 4A & 4B).

Table 1 Activity spectrum of the pure compound from *B. gladioli* OR1 against various drug resistant clinical isolates of *S. aureus.*

S. No.	Test strain (Source)	No. of strains	Resistance to drug/s	Zone of inhibition (diameter in mm; 1 mm = 10^{-3} m)
1.	*Staphylococcus aureus* (Umbilical tip)*	1	AMK, CIP, CLI, CLX, ERY, GEN, MET	14.0
2.	*S. aureus* (Pus/Semen)	2	ERY, GEN	14.0
3.	*S. aureus* (Wound)	1	GEN	14.0
4.	*S. aureus* (Pus/Wound)	3	CLX	14.0
5.	*S. aureus* (Wound)	2	AMK, CIP, ERY, GEN	13.5
6.	*S. aureus* (Tissue cells)	1	AMK, CLX, GEN	13.5

Note: * Strain selected for experimental observations.
The control sterile filter paper disc, loaded with 10 µl of methanol and air dried at room temperature, did not exhibit any antimicrobial activity.
AMK - Amikacin, CIP - Ciprofloxacin, CLI - Clindamycin, CLX - Clinafloxacin, ERY - Erythromycin, GEN - Gentamicin, MET - Methicillin.

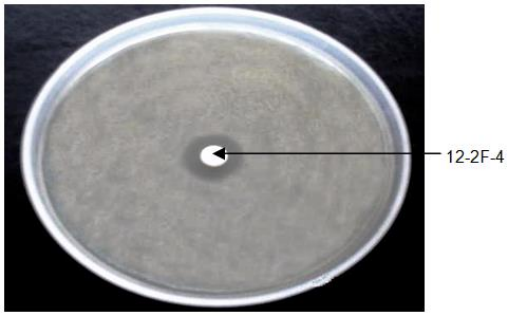


Figure 2 Zone of inhibition exhibited by the isolated pure compound 12-2F-4 against multi drug resistant *Staphylococcus aureus.*

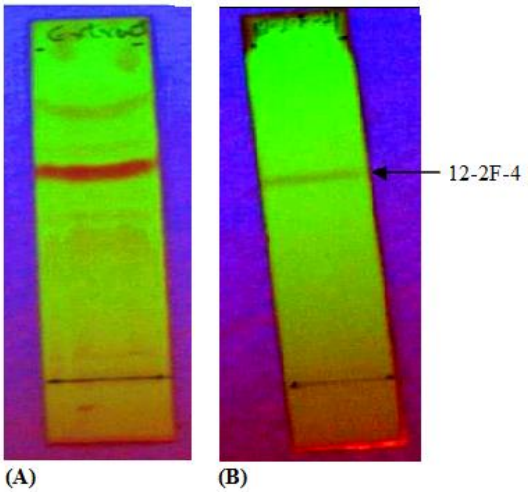


Figure 3 Comparative localization of bands on TLC (under UV light) in the (A) crude extract; (B) isolated spot 12-2F-4 obtained from *B. gladioli* OR1.

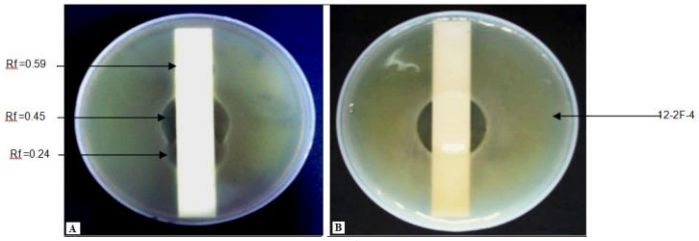


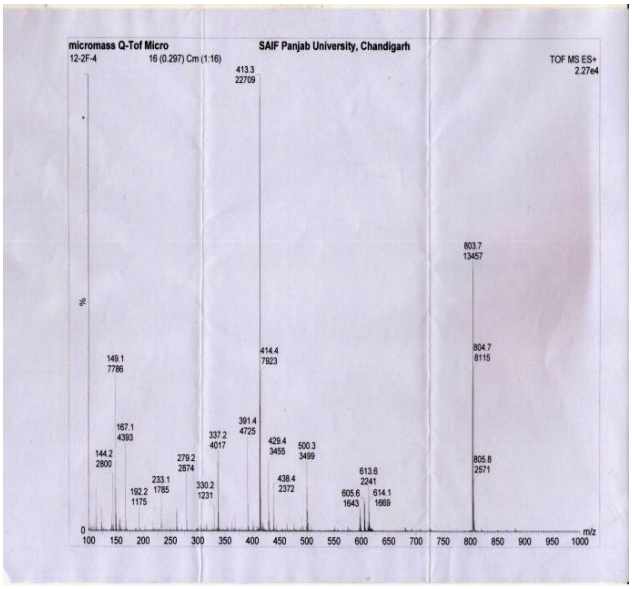
Figure 4 Comparative localization of anti- *S. aureus* zones upon TLC-bioautography analysis in the (A) crude extract; (B) isolated spot 12-2F-4.

Structural characterization of the pure active compound

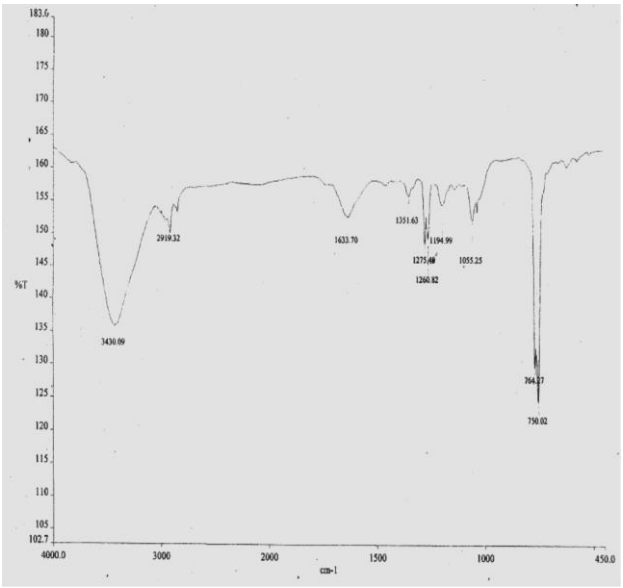
The positive mode APCI mass spectrum of 12-2F-4, showed $[M+1]^+$ molecular ion peak at *m/z* 391.4 suggesting that the molecular weight of the compound was 390 (Fig. 5A). IR spectrum of 12-2F-4 showed bands at 3430, 2919, 1633 and 1260, which indicated the presence of NH (OH), CH, C=C and C-O groups respectively (Fig. 5B).

The ^{1}H NMR spectrum of 12-2F-4 showed signals for two aromatic protons at δ 7.50 (2H, d, *J* = 2.8 Hz) and 7.41 (2H, d, *J* = 3 Hz). The spectrum also exhibited the presence of 10 methylene protons, 8 of which showed overlapping signals in the range δ 1.24-0.92 (8H, $4XCH_2$), and the remaining two protons at 3.99 (2H, $1XCH_2$). The signals for four methyl groups were in the range 0.74-0.69. A multiplet for one methine proton appeared at δ 1.46 (Fig. 5C).

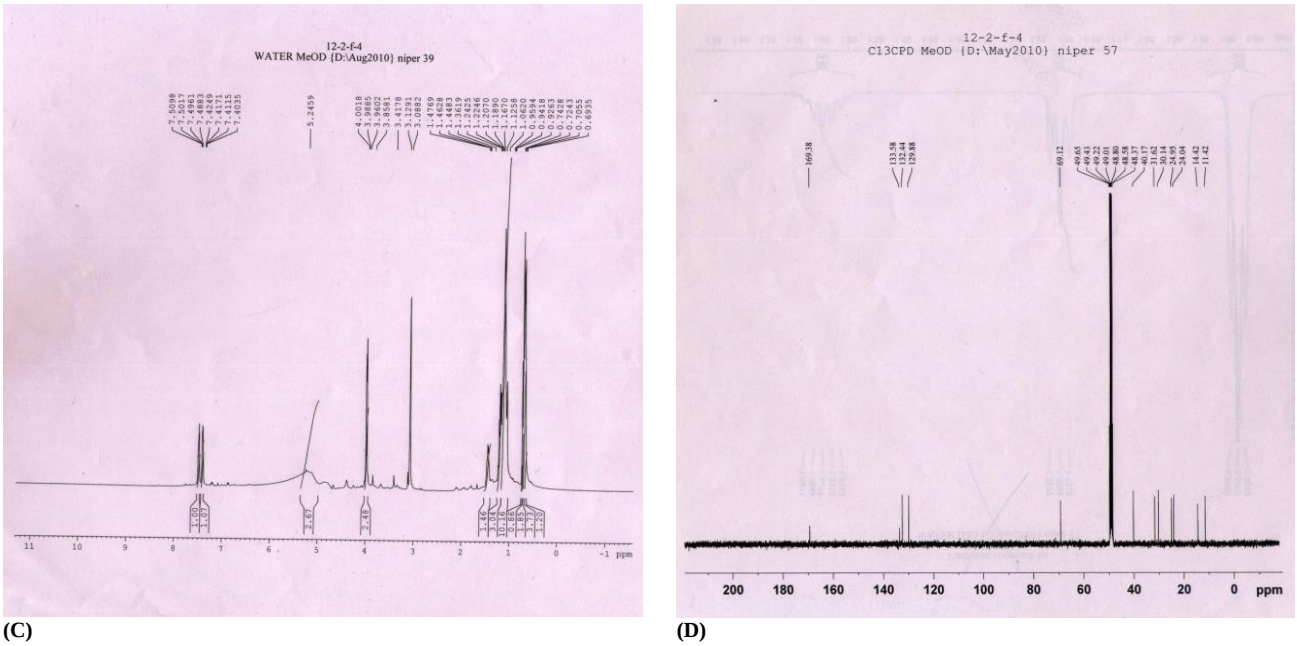
The ^{13}C NMR and DEPT spectral analysis of 12-2F-4 indicated 12 carbons at δ_C 169.4, 133.6, 132.4 129.9, 69.1, 40.2, 31.6, 30.1, 25.0, 24.0, 14.4 and 11.4. These were 4 terminal methyls (14.4 and 11.4), 2 aromatic carbons (132.4 and 129.9), 4 methylenes (31.6, 30.1, 25.0 and 24.0), 1 OCH_2 (69.1), 1 methine (40.2) and 2 quaternary carbons (169.4 and 133.6) (Fig. 5D).



(A)



(B)



(C) **(D)**

Figure 5 Spectroscopic data including (A) mass spectrum, (B) infra red spectrum, (C) ^{1}H nuclear magnetic resonance spectrum and (D) ^{13}C NMR spectrum of the pure active compound 12-2F-4.

The simplicity and clarity of the ^{1}H NMR and ^{13}C NMR spectra for 12-2F-4, led us to study its detailed structure using 2-D (dimensional) NMR {^{1}H-^{1}H COSY, HMQC, HMBC} spectroscopy. The ^{1}H-^{1}H-COSY spectrum of 12-2F-4 showed correlation between protons positioned at δ_H 3.99 and 1.46. The structure of the alkyl chain and substitution pattern at aromatic ring was confirmed by determining connectivity of C atoms from ^{1}H-^{13}C-HMBC spectrum; substitution pattern of benzene ring as confirmed by the ^{1}H-^{13}C-HMBC correlations and ^{1}H NMR splitting pattern led to fragment **A**, ^{1}H-^{13}C-HMBC correlations of protons at δ_H 3.99 and 1.46 with $2CH_2$ carbons indicated fragment **B** while ^{1}H-^{13}C-HMBC correlations and ^{1}H-^{1}H-COSY of terminal methyl groups present in the side chain confirmed the presence of 2 subunits B and **C** (Fig. 6).

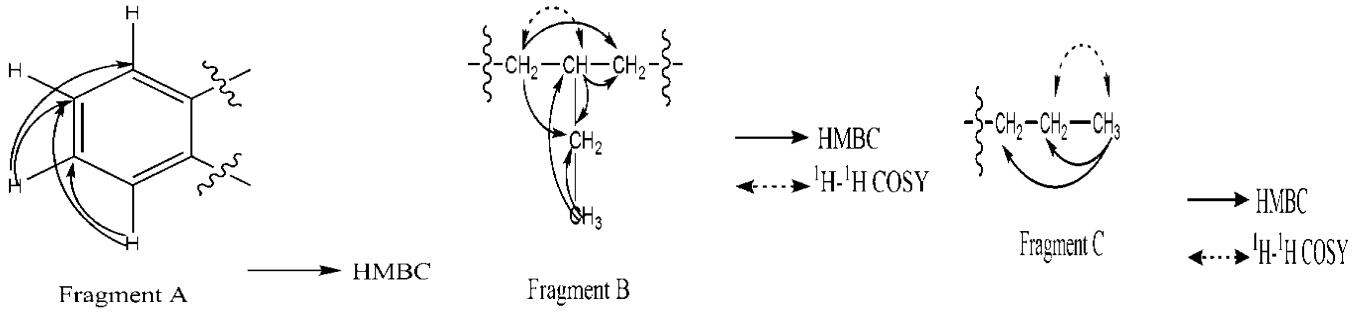


Figure 6 Major subunits of the pure active compound 12-2F-4 as elucidated by the spectral analysis.

The NMR data exhibiting the carbon resonances confirmed the symmetry of the molecule with formula $C_6H_4(C_8H_{17}COO)_2$. The final interpretation of all the combined spectral data suggested that the isolated pure compound from *B. gladioli* OR1 with anti-*S. aureus* activity is a symmetrical phthalate ester identified as bis-(2-ethylhexyl) phthalate (Fig. 7).

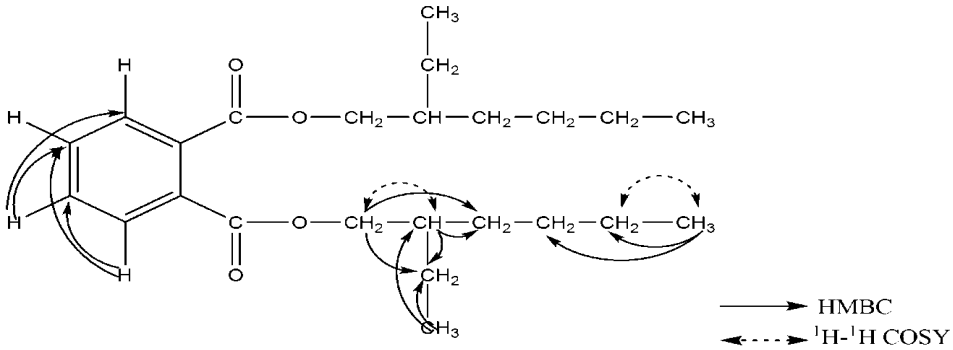


Figure 7 Chemical structure of the anti- *S. aureus* compound {bis-(2-ethylhexyl) phthalate}, purified from *B. gladioli* OR1.

DISCUSSION

In our earlier study, the chloroform extract containing antimicrobial compounds was found to inhibit the growth of many strains of antibiotic-resistant *S. aureus* (zone size: 15.0-33.0 mm or 0.015-0.033 m). The active extract revealed many bands in TLC analysis under UV light (254 nm and 366 nm) with R_f values 0.24, 0.45, 0.59, 0.62 and 0.76, and with three of them (corresponding to R_f values 0.24, 0.45 and 0.59) possessing anti-*S. aureus* activity (**Bharti *et al.*, 2011**). This led us to undertake the process of purification and characterization of active compounds possessing activity against MDR *S. aureus*. However, the compound responsible for biological activity is often present in trace amounts against a background of large amounts of biosynthetically related and structurally unrelated metabolites making the process challenging. The versatility, resolving capability, and quantitative accuracy of HPLC makes it one of the best direct methods to study the production of antibiotics *in situ* (**Raaijmakers *et al.*, 2002**). This is among a few reports on the complete characterization of an antimicrobial compound from *B. gladioli*. Only in the recent past, a single study has reported

the isolation of two bioactive volatile compounds, a liquid hydrocarbon cyclic terpene and a flavonone, with antifungal activity from *Burkholderia gladioli* pv. *agaricicola* (**Elshafie *et al*., 2013**). Earlier, **Box *et al*. (1988)** and **Gwynn *et al*. (1988)** reported the partial characterization of antimicrobial compounds belonging to the members of monobactam and bulgecin family of antibiotics from *B. gladioli*.

Moreover, this is the first report on the production of bis-(2-ethylhexyl) phthalate by any unicellular bacteria such as those belonging to the genus *Burkholderia*. This compound has been earlier isolated primarily from filamentous bacteria like *Nocardia levis* (**Kavitha *et al*., 2009**), S*treptomyces bangladeshiensis* (**Al-Bari *et al*., 2006**), *Streptomyces avidinii* (**Lyutskanova *et al*., 2009**), *Streptomyces* sp. TN17 (**Smaoui *et al*., 2011**), *Streptomyces mirabilis* NSQu-25 (**El-Sayed, 2012**) and other actinomycetes like *Saccharothrix sp.* (**Johnson *et al*., 2012**).

CONCLUSION

The incessant search for less toxic, more potent antibiotics, which are effective against the drug-resistant strains of major and opportunistic pathogens, led us to isolate and purify a pure compound from *B. gladioli* OR1. This compound identified as bis-(2-ethylhexyl) phthalate was found to exhibit potential activity against multi antibiotic resistant nosocomial pathogen *S. aureus*. *B. gladioli* can thus be looked upon as a potential source for the production of a novel class of antibiotics, effective against pathogens, resistant to the previously available anti-infective drugs. Further, chemical modification of purified anti-staphylococcal compound may be undertaken to enhance its antimicrobial activity.

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