In vitro antifungal activity of 9, 10-dihydrophenanthrene-2-carboxylic acid isolated from a marine bacterium: *Pseudomonas putida*

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Abstract: An antifungal compound 9, 10-dihydrophenanthrene - 2 - carboxylic acid was isolated from a marine derived bacterium *Pseudomonas putida* isolated from surface water samples of Karachi fish harbor coast line. The structure was explored using extensive 1D- and 2D-NMR spectroscopic techniques. The compound was found to be active against fungal strains obtained from clinical samples whereas strong activity was noted against *Candida albicans* with a MIC value of 20µg/ml, as the purified compound showed promising anticandidal activity a multidisciplinary approach is needed to explore further this compound as potential pharmacological lead compound against *Candida* spp and will add in the global hunt for clinically functional antifungal agents.

Keywords: Antifungal compound, marine bacteria, *Pseudomonas putida,* column chromatography, 1D and 2D-NMR spectroscopy.

INTRODUCTION

Fungal infections that are challenging to treatment are a budding community health dilemma as inadequate antifungal drugs are accessible in the market to treat infections caused by of life-threatening fungal strains (Fisher *et al*., 2012). Drug resistant *Aspergillus* and *Candida* are globally distributed and reported to cause infections in both in healthy and immune compromised individuals hence considered as rising menace to public health (Dagenais and Keller 2009; Arendrup 2014). The most widespread root of healthcare-associated bloodstream infections is mainly due to the fungus *Candida* (Magill *et al*., 2014). Strains of *Candida and Aspergillus spp* are reported as resistant strains to first and second-line antifungal medications have called attention to the requirement for developing innovative dominant antifungal against resistant fungal strains having negligible unfavorable side effects in humans (Ostrosky-Zeichner *et al*., 2010).

The marine milieu provides a precious niche for the isolation and discovery of new antifungal compounds approximately 4000 compounds active against various infectious agents have been isolated from marine environment (Hu *et al*., 2015). In our continued screening of bioactive constituents from marine derived *Pseudomonas* species (Uzair *et al*., 2008: Uzair *et al*., 2018) various species of this genus were isolated from marine samples producing bioactive compounds (Hu *et al*., 2015). In this paper the isolation, structure elucidation and antifungal activity of compound 1 against *Candida* and *Aspergillus* strains purified from crude extract of marine derived *Pseudomonas putida* is reported for the first time.

MATERIALS AND METHODS

Collection of sample for isolation of Antibiotic producing strain

A total of five marine surface water samples were collected from Karachi fish harbor coast line for the isolation of associated marine bacteria. From the collected samples morphologically different bacterial strains were isolated using serial dilution method on brain heart infusion broth and screened for antifungal activity by agar well diffusion method (Uzair *et al*., 2008) using *Candida* and *Aspergillus strains*. The strain showed positive antifungal activity was identified as *Pseudomonas putida.*

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General experimental procedures for structure revelation
The ^{1}H-NMR spectra were obtained using Bruker AMX-400 instrument operating at 400 MHz, The ^{13}C –NMR spectra were recorded on Bruker AMX-400 instrument operating at 100MHz. The chemical shift values are reported in ppm (δ) units and the coupling constants (J) are in Hz. Mass spectrum was recorded on Finningan MAT-312 double focusing mass spectrometers. precoated with silica gel on Aluminum sheets 60 F_{254} were used for Thin Layer chromatography, Column chromatography was performed using silica gel 60(70-230 mesh, Merck).

Fermentation of pseudomonas putida
Semi solid Brain heart infusion medium was used for the growth of selected *Pseudomonas putida*. The strain was inoculated in to 5 flasks of 2000ml capacity containing each containing 1000mL Brain heart infusion semi solid medium supplemented with 10% glycerol and cultured at 28˚C for five days on a rotary shaker at 150 rpm.

Extraction and isolation
The fermented brain heart infusion was filtered using cheese cloth to remove bacterial cells. The collected filtrate was extracted with methanol at room temperature in separating funnel. The methanol solution was evaporated using rotary evaporator and crude extract of about 10.5 gram was obtained from total culture broth of 5 liter. The slurry of crude extract was loaded on silica gel glass column for compound purification (70~230 mesh size of silica). A gradient of ethyl acetate in hexane was used as mobile phase for the separation of compounds from the loaded crude extract. The fractions eluted with hexane - ethyl acetate showed the presence of one main yellow colored compound. The fractions collected from the small column eluted in hexane - ethyl acetate (9:1) purified compound 1 in pure form as yellow colored compound.

Antifungal activity and MIC determination of purified compound
Disc diffusion method was used as an antimicrobial method (Bordoloi, *et al*., 2001). Sterile -SDA-(Oxoid CM0041) at 43 - 45ºC was poured into the petri plates (9 cm diameter). Test fungal culture inoculum was applied on each Sabouraud Dextrose Agar agar plate, the inoculum was spreaded on agar medium using spread plate technique.. Sterile filter papers discs of 6 mm diameter impregnated with 50µL compound 1, between 50 - 900 mg/mL concentrations were placed on SDA agar plates and the inoculated plates were incubated at room temperature and observed daily for the formation of zones of inhibition around discs and sizes of zones of inhibition were measured in mm. MIC of purified compound was determined using broth dilution method as directed in National Committee for Clinical Laboratory Standards (NCCLS 1997).

RESULTS

Isolation and identification of Antibiotic producing strain
Antifungal compound producing *Pseudomonas putida* strain was isolated from marine water sample collected from Karachi fish harbor the isolated strain was identified using standard biochemical tests of API 20 NE kit.

Preparation of crude extract
Crude extract was obtained from semi solid culture medium consist of brain heart infusion broth supplemented with 10% glycerol. A total of 10.5gram crude extract was obtained from 5 liter growth medium using organic solvent methanol.

Isolation and purification of compound
A yellow colored compound was purified from silica gel glass column and its purity was double checked on TLC and HPTLC cards. A purified band of yellow colored compound is clearly visible on TLC card (fig. 1) these eluted compounds were pooled in one test tube and used for further experiments.

Antifungal activity of purified compound 1
Antifungal activity against *Candida* spp and *Aspergillus* clinical strains was screened using disc diffusion method (fig. 1). As indicted in table 1 compound 1 showed strong activity against all tested *Candida* and *Aspergillus* species (fig. 1).

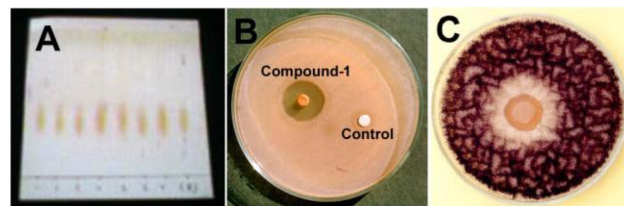

Fig. 1: Purification and antifungal activity of compound - 1 against fungal strain Key; A: TLC card showing detection of yellow colored compound-1 in pure form, B; anti *Candidal* activity of compound-1, C; Anti - *Aspergillus* activity of compound-1

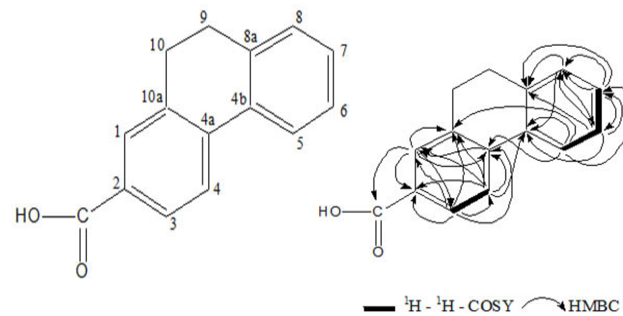

Fig. 2: Chemical structure of compound-1, ^{1}H-^{1}H-COSY and HMBC interactions.

Table 1: *In vitro* antifungal activity of compound -1 by disc diffusion assay

Test fungi	Fungal strain source	Size of Zone of inhibition (mm	Minimum inhibitory concentration (μg ml^{-1})
Candida albicans	Blood specimen	24.4	20
Candida albicans	Urine sample	24.6	20
Candida tropicalis	Urine sample	22.0	30
Aspergillus fumigatus	Lung biopsy	15.4	50
Aspergillus flavus	Pleural fluid	16.4	44
Aspergillus niger	Skin sample	14.5	45

Table 2: ^{1}H-NMR and ^{13}C-NMR spectral readings of compound -1

C/H #	^{13}C	DEPT	HMQC	Multiplicity	HMBC correlations
1	130.48	CH	8.66	dd	C-2, C-4a,C-10a,-COOH
2	128.89	C	-	-	-
3	132.34	CH	8.38	dd	C-1,C-2,C-4,C-4a,C-10a
4	128.88	CH	7.05	br.d	C-1,C-2,C-3,C-4a,C-4b,C-10a
4a	140.83	C	-	-	-
4b	133.54	C	-	-	-
5	119.11	CH	8.01	m	C-3,C-4a,C-4b, C-7,C-8,C-10a
6	128.68	CH	8.32	m	C-7,C-8,C-4a,C-4b,C-8a
7	122.16	CH	7.80	m	C-6,C-8,C-4a,C-4b,C—8a
8	129.75	CH	7.85	m	C-6,C-7,C-8a,C-4a,C-4b
8a	143.19	C	-	-	-
9	29.68	CH_2	1.23	s	-
10	29.68	CH_2	1.23	s	-
10a	151.54	C	-	-	-
COOH	176.60	C	10.69	s	-

Structure elucidation

The molecular formula of $C_{15}H_{12}O_2$ was obtained of compound 1 by Electron impact mass spectrum representing peak at m/z 224 of compound 1. The IR spectrum (KBr) absorption band at 3330 cm^{-1} showing the presence of OH group, The ^{1}H-NMR spectrum depicted aromatic protons from C-1, to C-8 as shown in Table 2. The acidic proton of-COOH appeared at δ 10.68. ^{13}C-NMR spectrum in $CDCl_3$ showed the presence of 15 carbon resonances in accord to the molecular formula of $C_{15}H_{12}O_2$. The ^{13}C-NMR spectrum (table 2) revealed occurrence of seven aromatic carbon signals at δ 135.98(C-1), 134.36(C-3), 129.88(C-4), 129.11(C-5), 129.79(C-6), 131.06(C-7) and 131.75(C-8). The methylene signal was observed at δ 29.68 due to C-9 and C-10. The appearance of a signal at δ166.60 is due to the presence of CO_2H group. One-bond correlations between hydrogen and carbon were analysed by HMQC provided definite confirmation to establish the structure of compound 1(fig. 2). The aromatic proton signal of C-1, C-3, C-4, C-5, C-6, C-7 and C-8 in the ^{13}C-NMR spectrum at δ 135.98, 134.36, 129.88, 129.11, 129.79, 31.06 and 131.75 and the structure of compound-1 was additionally established by 2D ^{1}H-^{1}H Homo Nuclear Chemical Shift Correlation spectroscopy (COSY-45˚) (Atta-ur-Rehman, 1989) which showed the connectivity of H-3 (δ 8.41) and H-4 (δ 7.95). Hence using ^{13}C-NMR, ^{1}H-NMR, COSY, HMQC and HMBC spectra the structure of compound 1 was ascertained as 9, 10 dihydrophenanthrene - 2 - carboxlic acid (fig. 2).

Here we first time report isolation of compound from marine water associated bacterium *Pseudomonas putida* although its synthesis has been reported in the literature (Abell *et al*., 1997) but its natural isolation is reported here for the first time.

DISCUSSION

One way out to the collective catastrophe of AMR is the detection of new antimicrobial compounds for medical request. Particularly New antifungal agents are in high need to combat infections caused by drug resistant fungal infectious agents (Goffeau 2008). To explore new antifungal compounds marine environment is considered as important niche for the isolation of new target compounds of antifungal nature as previously marine environment is turned out to be important avenue for antimicrobial agents (Thomas *et al*., 2010). In this study we have isolated strain of *Pseudomonas putida* from marine surface water sample collected from coast line of Karachi fish harbor. Previously we have reported strain of

kouria marina showing both antibacterial and antifungal activity (Uzair *et al*., 2018). The strain was grown best on brain heart semi solid medium for the growth and subsequent purification of the compound from the crude extract obtained after fermentation. A yellow colored antifungal compound 9, 10-dihydrophenanthrene - 2 - carboxylic acid was purified from crude extract of *Pseudomonas putida* using silica gel glass column chromatography technique having molecular formula of $C_{15}H_{12}O_2$. Various antifungal compounds were reported previously from marine habitat such as Basiliskamides A (12) and B (13) showing anticandidal activity from marine based *Bacillus laterosporus* (Barsby *et al*., 2002). In this study a potent antifungal compound is reported from marine based *Pseudomonas* strain previously various antimicrobial compounds from *Pseudomonas* strains are reported indicating this genera as important reservoir for obtaining antimicrobial secondary metabolites search for antifungal drug is always challenging as fungal cells are analogous to mammalian cells required specific target compound that only kill infection causing fungal strains. Discovery of new antifungal agents with novel mode of action is a burning medical requisite because of two main reasons one is development of resistance against existing agents and second due to side effects of currently known antifungal agents. The marine environment is considered as an untapped tank which is yet not explored fully as a resource of natural antibiotics with functional and structural diversity Many marine based anti infective compounds have entered phase I, II and III clinical trials (Mayer *et al*., 2010; Martins *et al*., 2014) but unluckily, none of them are of antifungal nature so we are in dire need to search new antifungal agents.

CONCLUSION

In this study we report isolation and characterization of a new antifungal compound active against various fungi of medical importance; we found marine environment could be a reservoir for the isolation of diverse marine bacterial strains having unique secondary metabolites to be used as lead compounds for controlling fungal strains

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