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Phialomustin A-D, new antimicrobial and cytotoxic metabolites from an endophytic fungus, Phialophora mustea.

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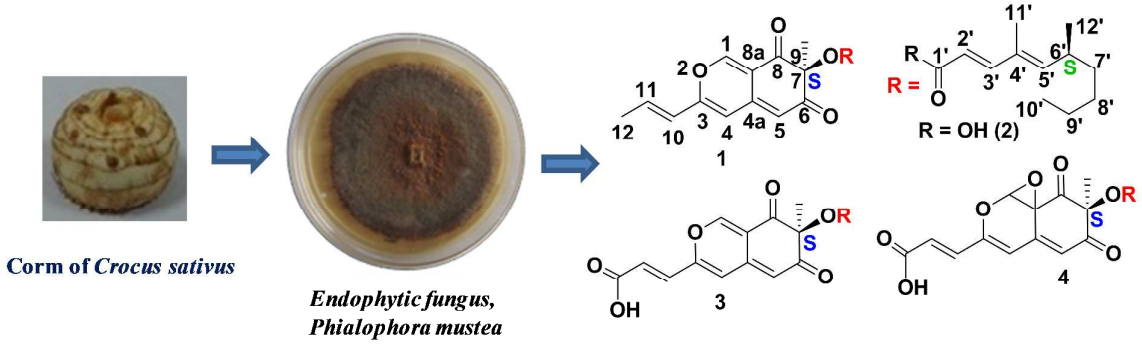
Graphical abstract

Phialomustin A-D (**1-4**), four new azaphilone derived bioactive metabolite, were isolated from an endophytic fungus *Phialophora mustea* associated in nature with *Crocus sativus*. The structural determinations of compounds (**1-4**) were authenticated by spectroscopic and chemical analysis. Compounds **3** and **4** showed promising antifungal activities with IC_{50} values of 14.3 and 73.6 µM, against *Candida albicans,* whereas compound **2** exhibited remarkable cytotoxic potential against the human breast cancer cell line, T47D with an IC_{50} of 1 µM.



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Phialomustin A-D, new antimicrobial and cytotoxic metabolites from an endophytic fungus, *Phialophora mustea*

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Phialomustin A-D (**1-4**), four new bioactive metabolite, with an unprecedented azaphilone derived skeleton, were isolated and characterized from an endophytic fungus isolated from *Crocus sativus.* The ITS-5.8S-ITS2 ribosomal gene sequence of the endophte displayed a sequence similarity of more than 99% with *Phialophora mustea.* The structural determinations of compounds (**1-4**) were authenticated by spectroscopic and chemical analysis. The absolute configuration of stereogenic centers of **1,3-4** were determined by electronic circular dichroism spectroscopy. Compounds **3** and **4** showed promising antifungal activities with IC_{50} values of 14.3 and 73.6 µM, against *Candida albicans,* whereas compound **2** exhibited remarkable cytotoxic activity against the Human breast cancer cell line, T47D with an IC_{50} of 1 µM.

Introduction

Fungal endophytes are a diverse class of microorganisms that colonize plants without causing any symptoms of disease. Endophytes have been isolated from the roots and aerial parts of a diverse range of hosts including algae [1], bryophytes [2], pteridophytes [3], gymnosperms [4], and angiosperms [5]. Fungal endophytes are one of the rich sources of novel bioactive secondary metabolites which display a broad spectrum of biological activities *viz.,* antimicrobial, immunosupressive, anticancer, antioxidant etc. [6-8] The proficiency of endophytes to produce diverse secondary metabolites inspired us to explore the isolation of bioactive metabolite from an endophytic fungus, *Phialophora mustea* isolated from *Crocus sativus*. Recent literature indicated that only 12 molecules were reported from the genus *Phialophora,* however, no such study has been carried out on *Phialophora mustea*. [9]

Recent studies have reported that azaphilone derived molecules exhibit wide range of biological functions, such as antimicrobial, antiviral, cytotoxic and anti-inflammatory [10, 11] as well as several bioactivities such as inhibition of gp120−CD4 binding [12], Grb2-SH2 interaction [13], MDM2−p53 interaction [14] and heat shock protein 90 (Hsp90) [15]. In the current study, structural elucidation of four new azaphilone derived metabolites (**1- 4**), from the crude extract of *Phialophora mustea,* an endophyte of Saffron (*Crocus sativus)* corms, was carried out. Further, these compounds were evaluated for cytotoxic and antimicrobial activities.

Results and Discussion

The fungal isolate, CS7E2 was found associated as an endophyte with the corms of *Crocus sativus*. Aquistion of the ITS-5.8S-ITS2 ribosomal gene sequence showed that the organism is a strain of *Phialophora mustea.* The nucleotide sequence showed more than 99% homology with *Phialophora mustea* IFM 50365 (GenBank: AB190404.1). The evolutionary position of CS7E2 is presented in Fig S1(supporting information). The culture broth (25 L) of CS7E2 was extracted with EtOAc after fermentation for 10 days. Among the common fungal media used, maximum growth and quantity of secondary metabolites were observed in the potato dextrose broth. The conditions used during the fermentation yielded similar growth characteristics as in the shake flask. The 6.0 g crude extract, thus obtained, was subjected to flash column chromatography on silica gel (230-400 mesh) which led to the isolation of compounds **1-4.**

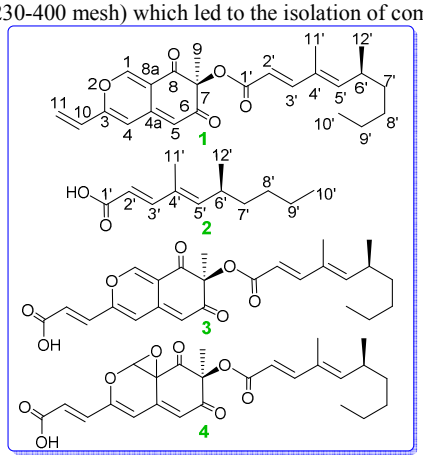

Figure 1: Structure of phialomustin A-D (**1- 4**)

Compound **1** was obtained as yellow coloured viscous oil having $[\alpha]_D^{25}$ + 510 (c 2.0, $CHCl_3$). Its molecular formula, $C_{25}H_{30}O_5$, was deduced from the positive ion HRESIMS at *m/z* 411.2170 $[M+H]^+$ (see SI Fig. S9) and ^{13}C NMR spectroscopic data, indicating 11 indices of hydrogen deficiency. A close inspection of the ^1H and ^{13}C NMR spectra of **1** by DEPT and HSQC experiments revealed the presence of three carbonyl (C-6, C-8 and C-1′), four sp^2 quaternary (C-3, C-4a, C-8a, C-4′), eight sp^2 methine (C-1, C-4, C-5, C-10, C-11, C-2′,C-3′, C-5′), one sp^3 quaternary (C-7), one sp^3 methine (C-6′), three sp^3 methylene (C-7′, C-8′, C-9′), and five methyl carbons

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[#] Electronic supplementary information (ESI) available: NMR spectra of all new compounds. See DOI: 10.1039/x0xx00000x

(C-9, C-12, C-10′, C-11′, C-12′). The ^{1}H-^{1}H COSY analysis of **1** led to three partial structural units, H-10 (δ 6.01, d, *J* = 15.6 Hz) ↔ H-11 (δ 6.55, m) ↔ H-12 (δ 1.93, d, *J* = 6.8 Hz), H-2′ (δ 5.92, d, *J* = 15.6 Hz) ↔ H-3′ (δ 7.36, d, *J* = 15.7 Hz), H-11′ (δ 1.78, s) (allylic) ↔ H-5′ (δ 6.01, d, *J* = 9.7 Hz)↔ H-6′ (δ 2.51, m) ↔ H-7′ (δ 1.38 – 1.30, m) ↔ H-8′ (δ 1.27, m) ↔ H-9′ (δ 1.23 – 1.18, m) ↔ H-10′ (δ 0.87, t, *J* = 6.9 Hz) and H-6′ (δ 2.51, m) ↔ H-12′ (δ 0.97, d, *J* = 6.6 Hz) as shown by bold faced lines in (Figure 3). The HMBC spectrum showed key correlations of H-10/C-3, C-4, C-11 and C-12, of H-4/C-8, C-6, C-3, C-4a, C-8a, C-10 and C-5, of H-5/C-7, C-4 and C-8a, of H-1/C-8, C-3, C-4a and 8a, of H_3-9/C-8, C-6, C-7 (Table 1 and Figure 3). These spectral evidences clearly indicated that azaphilone core moiety is similar to mitorubrin [16]. The difference between **1** and mitorubrin was the side chain at C-7. The HMBC correlations of H-6′/C-4′, C-5′, C-7′, C-8′ and C-12′, of H-5′/C-3′, C-6′, C-7′, C-11′ and C-12′, of H-11′/ C-3′ and C-4′, of H-3′/C-1′, C-5′,C- 4′,C- 2′ and C- 11′, of H-2′/C-1′ and C-4′ clearly indicated that the compound **1** had 4,6-dimethyldeca-2,4-dienoic acid unit (Figure 3). The *E*-configuration was assigned to Δ2′,3′ and Δ4′,5′ alkene units on the basis of large coupling constant (*J* = 15.6 Hz) between H-2′ and H-3′, and NOESY correlations between H-2′/H-5′ and H-3′/ H-5′ and between H-11′/ H-12′ and H-5′/ H-7′ (Figure 3). Even though no direct HMBC and NOESY correlations were found between these two structural units, they were connected C-1′ to C-7 via ester linkage. This assumption was confirmed by chemical shift of C-7 (δ_C 83.94) which was further authenticated by ester hydrolysis of **1** with 4N NaOH which afforded **5** and **2** (Figure 2).

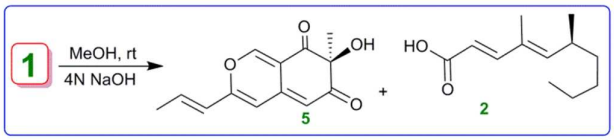

Figure 2: Hydrolysis of compound **1**

The absolute configuration of methyl at C-7 position of compound **1**,**3-4** were established by comparing its CD spectrum with those of azaphilone and sclerotiorin [17, 18] which showed positive (352 nm) and negative (286 nm) Cotton effects indicating the *S* configuration at C-7 (Figure 4). The lipid side chain **2** was identified as (+) (2*E*,4*E*,6*S*)-4,6-dimethyldeca-2,4-dienoic acid by comparing the NMR and specific rotation with literature; thus, absolute configuration at C-6′ was assigned as *S* [19].

Based on spectral and chemical evidences, the structure of the new molecule **1** was assigned as (*S*,2*E*,4*E*)-(*S*)-7-methyl-6,8-dioxo-3-(*E*)-prop-1-en-1-yl)-7,8-dihydro-6H-isochromen-7-yl 4,6-dimethyldeca-2,4-dienoate (figure 1) and it was named phialomustin A.

Compound **2** was obtained as colorless oil having $[\alpha]_D^{25}$ + 55.5 (c 1.5, $CHCl_3$). Its molecular formula, $C_{12}H_{20}O_2$, was deduced from the positive ion HRESIMS at *m/z* 197.1540 $[M+H]^+$ (See SI Fig. S15) and ^{13}C NMR spectroscopic data, indicating 3 indices of hydrogen deficiency. Compound **2** was identified as (+) (2*E*,4*E*,6*S*)-4,6-dimethyldeca-2,4-dienoic acid based on the comparison of ^{1}H , ^{13}C NMR and optical rotation data with those described in the literature [19] thus absolute configuration at C-6′ was assigned as *S*. The *R* (−) and *S* (+) of **2** were synthesized by Alcaraz *et. al* (1999) as a side chain acids for total synthesis of manumycin A, B and C [19]. To the best of our knowledge and literature database search we conclude that **2** is not a report as a natural product, which isolated from an endophytic fungus and was designated as phialomustin B (**2**).

Compound **3** was obtained as a yellow coloured amorphous powder having $[\alpha]_D^{25}$ + 525 (c 2.0, $CHCl_3$). Its molecular formula, $C_{25}H_{28}O_7$, was deduced from the positive ion HRESIMS at *m/z* 441.1921 $[M+H]^+$ (See SI Fig. S24) and ^{13}C NMR spectroscopic data, indicating 12 indices of hydrogen deficiency. The ^{1}H and ^{13}C spectral data of **3** were quite similar to **1**, except missing of one olefinic methyl and addition of one carbonyl group (δ 169.84) (Table 1). It indicates that allylic methyl was replaced by carboxylic acid group in **3**. It was further authenticated by the HMBC correlations of H-10/C-12, C-11, C-3 and C-4, of H-11/C-12, C-3, and C-10 (fig. 3). Thus, the detailed inspection of NMR (^{1}H, ^{13}C, DEPT, 2D) data, revealed the structure of new molecule **3**, as (*E*)-3-((*S*)-7-(((*S*,2*E*,4*E*)-4,6-dimethyldeca-2,4-dienoyl)oxy)-7-methyl-6,8-dioxo-7,8-dihydro-6H-isochromen-3-yl)acrylic acid, and this molecule was termed as phialomustin C.

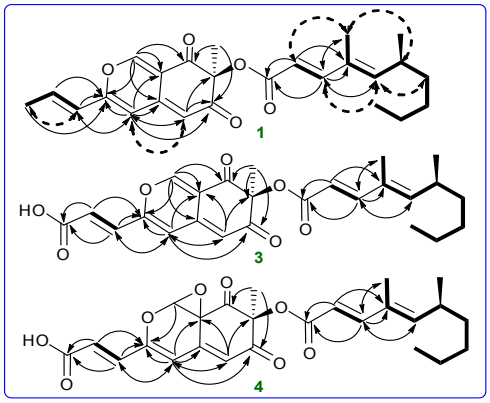

Figure 3: Key ^{1}H- ^{1}H COSY (▬), HMBC (◠) and NOESY (◡) correlations of compounds phialomustin A, B and D (**1**, **2** and **4**)

Compound **4** was obtained as a yellow amorphous powder with $[\alpha]_D^{25}$ + 440 (c 2.0, $CHCl_3$). Its molecular formula, $C_{25}H_{28}O_8$, was deduced from the positive ion HRESIMS at *m/z* 457.1857 $[M+H]^+$ (See SI Fig. S34) and ^{13}C NMR spectroscopic data, indicating 12 indices of hydrogen deficiency. The molecular formula of **4** contained one additional oxygen atom compared to **3**. The ^{1}H and ^{13}C NMR spectral data was quite similar to compound **3**, except for the loss of two olefinic carbons signals and the presence of two additional oxygenated carbons (δ_c 79.15, δ 54.80). It concludes that one of the double bonds in azophilone core was transformed by an epoxy group. Furthermore, it was authenticated by HMBC correlations of H-1/C-3, of H-4/C-8a, C-6, C-3, C-4a, C-10 and C-5, of H-5/C-8a, C-7 and C-4 (Table 1 and Figure 3). The stereochemistry of epoxide was not determined yet [20]. Based on 1D and 2D NMR spectral evidences, the structure of new compound **4** was assigned as (*E*)-3-((7*S*)-7-(((*S*,2*E*,4*E*)-4,6-dimethyldeca-2,4-dienoyl)oxy)-7-methyl-6,8-dioxo-1a,6,7,8- tetrahydroxireno[2,3-]isochromen-3-yl)acrylic acid and was termed as phialomustin D.

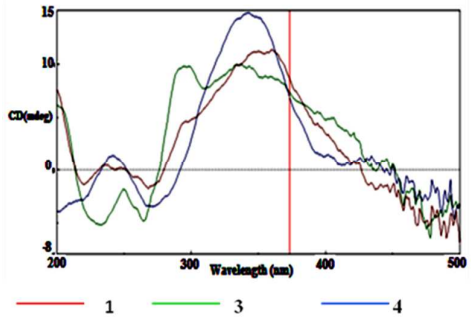

Figure 4: Comparison of experimental ECD spectra of **1** (red line), **3** (green line) and **4** (blue line).

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Table 1: NMR data of phialomustin A, C, D (**1**, **3**, **4**)

No.	1			3			4		
	δ_H (J in Hz)	δ_C [b]	HMBC	δ_H [a] (J in HZ)	δ_C [c],	HMBC	δ_H [a] (J in HZ)	δ_C [c], type	HMBC
1	7.91 s	153.5, CH	8, 3, 4a, 8a	7.93 s	153.4, CH	8, 3, 4a, 8a	5.94 s	79.1, CH	3
3		155.3, C			152.5, C			150.9, C	
4	6.11 s	108.6, CH	8, 6, 3, 4a, 8a, 10, 5	6.55 s	116.7, CH	8, 3, 10, 8a, 5	6.03 s	109.6, CH	8a, 6, 3, 4a, 10, 5
4a		142.7, C			141.0, C			143.2, C	
5	5.61 s	107.7, CH	7, 4, 8a	5.76 s	110.6, CH	7, 4, 8a	6.34 s	124.7, CH	8a, 7, 4
6		192.8, C			192.7, C			195.2, C	
7		83.94, C			84.0, C			83.8, C	
8		193.3, C			193.4, C			191.8, C	
8a		114.9, C			114.9, C			54.8, C	
9	1.58 s	22.29,CH_3	8, 6, 7	1.58 s	22.0, CH_3		1.65 s	22.0, CH_3	6, 8, 4′
10	6.01 d (15.6)	122.4, CH	3, 11, 4, 12	7.22 d (15.6)	135.2, CH	12, 11, 3, 4	7.05 d (15.4)	137.2, CH	12, 3, 11, 4
11	6.55 m	135.3, CH	3, 12	6.51 d (15.6)	123.6, CH	12, 3, 10	6.43 d (15.4)	123.4, CH	12, 3, 10
12	1.93 d (6.8)	18.5, CH_3	3, 11, 10, 4		169.8, C			170.5, C	
1′		166.3, C			166.5, C			166.3, C	
2′	5.92 d (15.6)	113.5, CH	1′, 4′	5.90 d (15.6)	113.1, CH	1′, 3′, 4′	5.81 d (15.6)	112.5, CH	1′, 3′, 4′
3′	7.36 d (15.7)	152.4, CH	1′, 5′, 4′, 2′, 11′	7.36 d (15.6)	152.9, CH	11′, 1′, 5′, 4′, 2′	7.30 d (15.6)	153.4, CH	1′, 5′, 4′, 2′, 11′
4′		131.4, C			131.4,C			131.4, C	
5′	5.67 d (9.7)	149.9, CH	3′, 11′, 12′, 7′, 6′	5.68 d (9.6)	150.5, CH	3′, 7′, 6′, 12′, 11′	5.65 d (9.7)	151.1, CH	3′, 2′, 7′, 6′, 12′, 11′
6′	2.51 m	33.2, CH	5′, 4′, 7′, 12′, 8′	2.50 m	33.3, CH	5′, 4′, 7′, 12′	2.45 s	33.4, CH	5′, 4′, 7′, 8′, 12′
7′	1.38 – 1.30 m	36.9, CH_2	5′, 8′, 6′, 9′, 12′	1.33 m	36.8, CH_2	9′, 8′	1.30 m	36.8, CH_2	5′, 6′, 8′, 9′
8′	1.27 m	29.7, CH_2	7′, 10′, 9′	1.25 m	22.7, CH_2	7′, 9′, 10′	1.21 – 1.16 m	29.7, CH_2	7′, 9′, 10′
9′	1.23 – 1.18 m	22.7, CH_2	7′, 10′	1.20 m	29.7, CH_2	7′,8′, 10′	1.16 – 1.10 m	22.7, CH_2	8′, 10′
10′	0.87 t (6.9)	14.0, CH_3	8′, 9′	0.85 t (6.8)	14.0, CH_3	9′, 8′, 5′	0.80 t (7.0)	14.0, CH_3	8′, 9′
11′	1.78 s	12.2, CH_3	3′, 4′	1.77 s	12.3, CH_3	3′, 5′, 4′	1.71 s	12.2, CH_3	3′, 5′, 4′
12′	0.97 d (6.6)	20.4, CH_3	5′, 7′, 6′	0.96 d (6.4)	20.4, CH_3	7′, 6′, 5′	0.90 d (6.5)	20.4, CH_3	5′, 7′, 6′

[a] ^{1}H NMR recorded at 400 MHz in $CDCl_3$. [b] ^{13}C NMR spectra at 100 MHz in $CDCl_3$. [c] ^{13}C NMR spectra at 125 MHz in $CDCl_3$

In addition compounds (**1-4**) along with the crude ethyl acetate extract were screened for antifungal and cytotoxic activities in four cancer cell line *viz* MIAPaCa2, A549, HCT-116 and T47D. The new compound **2** is an unsaturated lipid class of compound this is not to be known for cytotoxic potential so far exhibited the most promising cytotoxic potential against breast cancer cell line (T47D) with the IC_{50} values 1 µM.

Antifungal activities: Compounds **1-4** were evaluated for antimicrobial activities in which **3** and **4** showed potent antifungal activities with IC_{50} values of 14.3 and 73.6 µM, respectively, against the fungal pathogen, *Candida albicans* (MTCC 4748). However, no activity was found against the Gram negative bacterium, *Escherichia coli,* and the Gram positive bacteria *Staphylococcus aureus* and *Bacillus cereus,* up to concentrations of 100 µM. Furthermore,

Compound **3** was found active against the human pathogen *Aspergillus fumigatus*, and aflatoxin producing fungi, *Aspergillus parasiticus* and *Aspergillus flavus*, with IC_{50} values of 60.6, 35.2 and 84.4 µM, respectively (Table 2). However, no significant activity was found gainst the plant pathogens, *Fusarium oxysporum, Geotrichum candidum* and *Colletotrichum capsici* up to concentrations of 100 µM.

Table 2: Antifungal activity of Phialomustin C (**3**) against susceptible fungi. Nystatin was used as a positive control.

Compd. code	**IC_{50} µM**			
	CA	**AFu**	**AP**	**AFl**
Phialomustin C **(3)**	**14.3**	60.6	35.2	88.4
Nystatin (control)	1.6	1.7	1.7	2.8

CA (*Candida albicans* MTCC 4748), AFu (*Aspergillus fumigatus* MTCC 343), AP *(Aspergillus parasiticus* MTCC 2996), AFl *(Aspergillus flavus* MTCC 3380). However, phialomustin C did not display any significant activity against the bacterial pathogens *Escherichia coli* ATCC 25922*, Staphylococcus aureus* ATCC 29978 and *Bacillus cereus* IIIM 25, and the plant pathogens, *Fusarium oxysporum* MTCC 1755*, Geotrichum candidum* MTCC 3993 and *Colletotrichum capsici* MTCC 2071 up to a concentration of 100 µM. Ciprofloxacin was used as a positive control for bacterial inhibition.

Inhibition of cancer cell proliferation by isolated molecules: Phialomustin A-D, were screened for cytotoxic activity against four distinct human cancer cell lines including, pancreatic (MIA PaCa-2), lung (A549), colon (HCT 116) and human breast cancer (T47D), using the MTT method [21]. Interestingly, compound **2**, which is an unusual unsaturated fatty acid having two double bond at C-3, C-5 and two methyl at C-4, C-6 exhibited potent cytotoxic potential in T47D cell lines (IC_{50} 1 µM), while other isolates **1,3-4** also exhibited promising cytotoxicity (IC_{50} ~7-10 µM in T47D). The cytotoxic results of all the compounds (**1-4**) are summarised in (Table 3).

Table 3: *In vitro* cytotoxicity (IC_{50} values) of phialomustin A-D (**1-4**) against cancer cell lines

	MIAPaCa2	**A549**	**HCT-116**	**T47D**
C.E	20 ± 4.82	69 ± 3.63	100 ± 0.69	50 ± 4.19
1 (µM)	35 ± 1.19	98 ± 4.96	8 ± 3.2	10 ± 1.81
2 (µM)	70 ± 0.99	30 ± 4.15	30 ± 2.74	**1** ± 2.49
3 (µM)	38 ± 1.18	nd	100 ± 2.0	7 ± 1.59
4 (µM)	60 ± 2.65	nd	30 ± 0.92	9.2 ± 1.38
P (nM)	6.8 ± 0.020	2.5± 0.010	6 ± 0.027	11 ± 0.045

C.E = crude extract, P = Paclitaxel

Furthermore, the apoptosis induction by phialomustin B (**2**) in human breast cancer (T47D) cells was checked through Hoechst staining. Apoptotic bodies' formation increases in concentration dependent manner from (1–10 µM) for 24 h [22]. As evident by blebbing and apoptotic bodies (indicated by arrows in Figure 5), cells treated with **2** showed altered cellular morphology including cell wall deformation, shrinkage of cell size and nuclear condensation, whereas the nuclei of untreated cells were round and healthy.

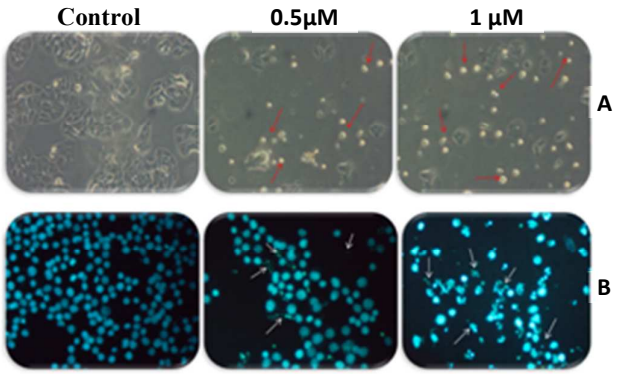

Figure 5: Effect of Phialomustin B (**2**) on the apoptosis and early events associated with it. (A) and (B) Effect of HMC on cellular and nuclear morphology. T47D cells were seeded in a 12 well plate, treated with indicated concentrations of **2** for 24 h and visualized for cellular and nuclear morphology.

Conclusions

In conclusion, phialomustin A-D (**1-4**) are new azaphilone derived molecules isolated from an endophytic fungus *Phialophora mustea.* Compounds **1-4** were screened for the first time for antifungal and cytotoxic potential in which the compounds **3** showed promising antifungal activities with an IC_{50} value of 14.3 µM against *Candida albicans* whereas the compound **2** exhibited remarkable cytotoxic activity against the human breast cancer cell line, T47D with an IC_{50} of 1 µM. Compound **2** is an unsaturated skeleton which has never been reported as cytotoxic agent. It may be explored further for the development as an anti-cancer lead metabolite for breast cancer.

Experimental:

General Experimental Procedure: Melting points of synthesised compounds were recorded on electrical melting point apparatus (Buchi, D-545) and are uncorrected. High resolution mass spectra were obtained on Agilent 6540 (Q-TOF) high resolution mass spectrometer, in the electrospray (ESIMS) mode. ^{1}H NMR spectra were recorded (Brucker Avance) DPX FT-NMR at 400 and 500 MHz and ^{13}C NMR at 125 MHz in $CDCl_3$ and CD_3OD, chemical shifts values are reported in δ (ppm) units and coupling constants values in hertz. Tetramethylsilane (TMS) was used as internal standard. Optical rotation was measured on a Perkin Elmer 341 polarimeter in a 1dm cell at 25°C. CD spectra were recorded using a JASCO J-810 CD spectrometer at a concentration of 1.0×10^{-4} M in MeOH at 25 °C. Both UV/vis and CD spectra were measured between 200 and 600 nm using 10 mm path-length quartz cuvettes. Infra-red spectra were recorded with a PerkinElmer spectrum 65 FT-IR spectrometer and wavenumber (n) are given in cm^{-1}. Column chromatography was performed using silica gel (230−400 mesh; Merck). Semipreparative HPLC was performed on an Agilent HPLC with a RP-18 column (250 × 4.6 mm, 5 µm; Agilent), a photodiode array detector and auto injector function (Agilent 1260 series). All the solvents were purchased from Merck. RPMI-1640, DMEM, 3-(4,5,-dimethylthiazole-2-yl)-2,5 diphenyltetrazolium bromide (MTT), Hoechst-33258 was purchased from Sigma-Aldrich (St Louis, MO). Fetal bovine serum was obtained from GIBCO Invitrogen Corporation USA. MIAPaCa-2 pancreatic cancer, A549 lung cancer, HCT-116 colon cancer and T47D human breast cancer cells were obtained from the National Cancer Institute (NCI), Bethesda, USA. The cells were grown in RPMI-1640 or DMEM medium supplemented with 10% heat inactivated fetal bovine serum (FBS), penicillin (100 units mL^{-1}), streptomycin (100 µg mL^{-1}), L-glutamine (0.3 mg mL^{-1}), pyruvic acid (0.11 mg mL^{-1}), and 0.37%

$NaHCO_3$. Cells were grown in a CO_2 incubator (Thermocon Electron Corporation, MA, USA) at 37 °C under an atmosphere of 95% air and 5% CO_2 with 98% humidity. Cells were treated with compounds dissolved in DMSO while the untreated control cultures received only the DMSO with concentration less than 0.2%. All microbiological media were procured from HiMedia, India.

Isolation and Fermentation

The endophytic fungus was isolated as described previously [23]. Corms of *Crocus sativus* were thoroughly washed with running tap water, and surface sterilized with 1% sodium hypochlorite and 70% ethanol. Before the treatment with alcohol, traces of sodium hypochlorite were removed by washing in sterile distilled water. The outer tissues were removed and the internal tissues were cut into small pieces of 0.5 to 1 cm and plated on water agar (Himedia, India). The plates were incubated at 25°C for three weeks. Hyphal tips of the fungus, emerging out of the plant tissue, were picked and grown on potato dextrose agar (HiMedia, India) in pure culture. The culture was also submitted to the National Fungal Culture Collection of India under the Voucher No. **NFCCI 3710.**

A week old culture medium (1%) was used to inoculate the 50 L stirrer tank reactor (Scigenic India Ltd), containing 25 L of potato dextrose broth (HiMedia, India). The fermentor was run with 100 RPM agitation at 25°C and 15 LPM air for 10 days.

Phylogenetic analysis by ITS1-5.8S-ITS2 ribosomal gene sequencing

Phylogenetic analysis of CS7E2 was carried out by the acquisition of the ITS1-5.8S-ITS2 ribosomal gene sequencing. The ITS regions of the fungi were amplified with the universal ITS primers, ITS4 (5′TCCTCCGCTTATTGATATGC3′) and ITS5 (5′GGAAGTAAAAGTCGTAACAA3′), using the polymerase chain reaction (PCR).[24] The amplified product was sequenced and aligned with the sequences in the GenBank by BLASTN program[25] to find out the sequence homology with closely related organisms. Sequences from the closely related organisms were downloaded and a phylogenetic tree was constructed. [26]

Determination of antimicrobial activities

The compounds isolated from CS7E2 evaluated for antimicrobial activities against a panel of pathogens. Stock solutions of each culture were prepared in normal saline solution (0.85% NaCl (w/v)) at a concentration of 10^8 cells/spores per ml. Mueller-Hinton (for bacteria; HiMedia, India) and potato dextrose broth (for fungi; HiMedia, India) were supplemented with the different concentrations of the compounds up to 200 µM. Each well was inoculated with 10^4 cells/spores of the relevant test organism and incubated at 37°C for 24 h. Absorbance was measured spectrophotometrically at 620 nm and IC_{50} of the extracts was calculated from the average percent inhibition of three replicates of each concentration. [27] Nystatin and Ciprofloxacin were used as positive controls.

Extraction and Isolation

25 liters culture broth of *Phialophora mustea,* extracted with ethyl acetate (EtOAc) and concentrated under reduced pressure, afforded 6 g of crude extract, which was subjected to column chromatography over silica gel (230−400 mesh) using a gradient of hexane and ethyl acetate (100:0 to 0:100) to give fractions (frs. 1−30). Phialomustin A (**1**) (27 mg) was isolated from fractions fr. 10 which was eluted with hexane-EtOAC (7:3). Furthermore, frs. 14-30 (4g) were pooled on the basis of similarities in TLC profiles and re-chromatographed over silica gel (230−400 mesh) with a step gradient of hexane and ethyl acetate (6:4). Frs. 6-8 yielded phialomustin B (**2**) (500 mg), obtained as a colourless and viscous substance. The column eluted with hexane-EtOAC (1:1) yielded phialomustin C (**3**) (2.3 g) and phialomustin D (**4**) (20 mg), both as yellow solids.

Phialomustin A (1): Yellow viscous oil; $[\alpha]_D^{25}$ + 510 (c 2.0, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 400 MHz) and ^{13}C NMR data, see Table 1; HRESIMS *m/z* 411.2170 $[M+H]^+$ (calcd for $C_{25}H_{31}O_5$ 411.2193).

Phialomustin B (2): Colourless viscous oil**;** $[\alpha]_D^{25}$ + 55.5 (c 1.5, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.43 (d, *J* = 15.6 Hz, 1H), 5.81 (d, *J* = 15.6 Hz, 1H), 5.75 (d, *J* = 9.8 Hz, 1H), 2.54 (dd, *J* = 14.5, 7.0 Hz, 1H), 1.82 (s, 3H), 1.50 – 1.15 (m, 6H), 1.01 (d, *J* = 6.5 Hz, 3H), 0.90 (t, *J* = 6.9 Hz, 3H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 173.01, 151.97, 149.75, 130.92, 114.31, 36.48, 32.91, 29.31, 22.40, 20.02, 13.65, 11.91; HRESIMS *m/z* 197.1540 $[M+H]^+$ (calcd for $C_{12}H_{20}O_2$ 197.1463).

Phialomustin C (3): Yellow amorphous powder; $[\alpha]_D^{25}$ + 525 (c 2.0, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 400 MHz) and ^{13}C NMR data, see Table 1; HRESIMS *m/z* 441.1921 $[M+H]^+$ (calcd for $C_{25}H_{29}O_7$ 441.1835).

Phialomustin D (4): Yellow amorphous powder; $[\alpha]_D^{25}$ + 440 (c 2.0, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 400 MHz) and ^{13}C NMR data, see Table 1; HRESIMS m/z 457.1857 $[M+H]^+$ (calcd for $C_{25}H_{29}O_8$ 457.1784).

Cell Proliferation Assay

The MTT assay was performed to determine the cell viability. Cells were seeded in 96 well plates and exposed to different concentrations of the compounds (**1-4**) for 48 h. The MTT dye (10 µl of 2.5 mg ml^{-1} in PBS) was added to each well 4 hours prior to experiment termination. The plates were then centrifuged at 1500 RPM for 15 min and the supernatant was discarded, while the MTT formazan crystals were dissolved in 150 µL of DMSO. The OD was measured at 570 nm [28].

Hoechst 33258 nuclear staining

Cells were treated with different concentrations of **2** (1–10 µM) for 24 h and washed twice with PBS at 400 × g for 5 min. Cells were then stained with 1 ml of staining solution (10 µg/ml Hoechst 33258, 0.01 M citric acid and 0.45 M disodium phosphate containing 0.05%Tween-20) and stained for 30 min in the dark at room temperature. After staining, the cells were resuspended in 50 µl of mounting fluid (PBS:glycerol, 1:1) and 10 µl mounting suspension was observed for nuclear morphology under inverted fluorescence microscope using UV excitation (Olympus 1X70, magnification 30X) [29].

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