Annual Academic Sessions 2011
ISSN 2012-9912; ©2011 Open University of Sri Lanka

A POTENTIAL ANTICANCER ACTIVE METABOLITE FROM SOIL FUNGUS *Penicillium citrinum*

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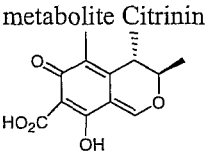
INTRODUCTION

Exploring the chemistry of fungal secondary metabolites had lead to the finding of a vast array of pharmaceuticals worldwide. As an initiation of discovering a novel potential anticancer drug candidate, a fractionation of a bioactive extract from *Penicillium citrinum* which is well known for the production of biologically active metabolite Citrinin **(1)**, was carried out.



(1)

A drug which is cytotoxic may have several disorders or side effects to the patients who receive the drug. Hence developing a screening method to target the anti cancer drug potential compounds, which are capable of inhibition or retardation of the cancer cell growth is a vital necessity. The Scratch wound assay which is presently used to measure cell migration *in vitro* can be modified and developed towards an anticancer assay. Herein we report the preliminary results of anticancer activity on scratch wound assay of a secondary metabolite from *P. citrinum*.

METHODOLOGY

A fungal culture (MW/01/04/03) isolated from soil and previously identified as a species of *Paecilomyces* using morphological characters, was used for the present study (Ranji *et al.* 2009).

Growing of Fungal culture

Broth cultures of the fungus were prepared by inoculating into a 500 mL conical flask containing 300 mL of the culture medium and keeping it on a rotary shaker for 21days. Solid cultures were prepared by inoculating the fungus into bottles (350mL) containing 40 mL of solid culture media and incubated at 30 °C for 21 days.

Molecular Identification

The fungal culture was grown in Yeast Malt Agar and the mycelium was used to extract DNA using Promega Extraction Kit. The ITS 1 and ITS 4 regions of the 18s-rDNA were amplified by Polymerase Chain Reaction (PCR) with the primers ITS 1 primer (5' TCCGTAGGTGAACCTGCGG) and ITS 4 primer (5'TCCTCCGCTTATTGATATGC). The PCR product was purified in order to carry out sequencing using the Geneshun kit for purification. Purified DNA was subjected to Automated Sequencing with the Big Dye Terminator Method and the resulted sequence was analyzed with the NCBI blast tool. (Hinrikson *et al.* 2005)

Large Scale Extraction of Secondary Metabolites

The culture grown in large scale (80 bottles each 350 mL) containing 40 mL of Potato Dextrose Agar (PDA) was extracted into ethyl acetate (EtOAc) as described below.

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Solid cultures were added with methanol (100 mL into each bottle) and shaken for 24 hrs on a rotary shaker and filtered. Filtrate was concentrated at 40 °C under vacuum to 1/5th of the original volume to remove methanol. The remainder was partitioned with EtOAc. The EtOAc extracts were combined and evaporated to dryness under vacuum at 40 °C to yield the fungal extract.

Fractionation and Isolation of Antibacterial Constituents

The EtOAc extract was dissolved in 80% aqueous methanol (MeOH) and partitioned between hexane. The aqueous MeOH layer was diluted with water to make a 50% aq. MeOH solution, and was then partitioned with dichloromethane (CH_2Cl_2). The solvents were removed under vacuum at 40 °C to yield the hexane fraction and CH_2Cl_2 fraction. A measured small volume of 50% aq. MeOH layer was also dried completely for the purpose of bioassay. All fractions were subjected to bioassay to find where the active constituents are located.

The CH_2Cl_2 fraction which showed antibacterial activity was subjected to further fractionation using silica column chromatography followed by preparative TLC separations to isolate the bioactive constituents.

Antibacterial Assay

Each and every extract/ fraction (dissolved in MeOH) was subjected to bioassay to determine antibiotic activity against several bacterial spp. (*Bacillus* sp., *E. coli, Staphylococcus* sp. and *Klebsiella* sp.) using Kirby-Bauer Disc Diffusion method (Pommerville and Alcamo 2004).

Scratch Wound Assay on Cancer Cells

Human cancer cell line, HEp 2 (adherent, monolayer) was established *in-vitro* in complete growth medium (Dulbecco's modified Eagle's medium, 10% fetal bovine serum), supplemented with antibiotics in plastic tissue culture flasks using standard methods.

Cells were transferred into 96 well plates with clear bottoms, incubated overnight at 37 °C with 5% CO_2 in a humidified incubator. After 12 hours (at confluence stage) a scratch was performed along the vertical axis of each well by inserting and pulling a sterile micropipette tip (2 – 20 µl) towards the user and scratch was washed with fresh medium (Yarrow *et al.* 2004).

From an initial investigation it was found that the appropriate concentration of DMSO to be used as the carrier of extracts, fractions and compounds without affecting the cell growth is 1% DMSO in growth medium.

Sample mixtures were prepared by dissolving the extracts / compounds in appropriate volumes of DMSO and mixed with complete growth medium (total volume 200 µl) and were introduced in to each well. 1% DMSO in growth medium was used as the control.

An inverted fluorescence microscope was used to visualize cells and the healing / inhibition of the scratch. Images were captured on a digital camera using a 10× objective. Plates were incubated for 24 hours and images were recaptured in the same way.

RESULTS AND DISCUSSION

Molecular Identification

Study of the molecular characters of the fungal culture (MW/01/04/03) led to the identification of the fungus as *Penicillium citrinum*.

Fractionation of the Ethyl Acetate Extract and Isolation of Antibacterial Constituents

The EtOAc extract of the fungus grown in large scale, showed antibacterial activity (Diameter of clear zone: 18 mm) against *Bacillus* sp. Hence this extract (1.27 g) was subjected to solvent partitioning followed by the bioassay of the resulted fractions at 500 µg per disc (See Table 1).

Fraction	Weight (g)	Dose (µg/disc)	Diameter of Clear Zone (mm)
Hexane	0.22	500	-
CH_2Cl_2	0.99	500	9.5
50% aqueous MeOH	0.03	500	-
Positive control: Amoxicillin		25	12

Table 1: Fractions obtained from solvent partitioning and their activity against *Bacillus* sp.

A pure compound was isolated by subjecting the CH_2Cl_2 fraction (905 mg) to a sequence of gel filtration chromatography and preparative thin layer chromatography. Isolated pure compound showed antibacterial activity against *Bacillus sp.* giving an average clear zone of 16 mm at a dose of 250 µg/ disc while amoxicillin (positive control) gave a clear zone of 12 mm at a dose of 25 µg/ disc.

Scratch Wound Assay on Cancer Cells

The EtOAc extract, Hexane fraction, CH_2Cl_2 fraction and the pure compound were subjected to scratch wound assay (See Table 2).

Fraction	Dose (mg / mL)	Growth effect
EtOAc extract	0.5	Inhibition
	1.0	Inhibition
	1.5	Toxic
	400	Toxic
Hexane	0.5	Toxic
CH_2Cl_2	0.5	Inhibition
Pure compound	0.5	Inhibition

Table 2: Results of Scratch Wound Assay

EtOAc extract at higher concentrations (≥1.5 mg / mL) and hexane fraction were found to be toxic to the cells thus detaching the cells from the surface. By comparing the width of the scratch, before and after incubation, it revealed that both the CH_2Cl_2 fraction as well as the pure compound (See Figure 2) had inhibited growth of the cells when compared to the control, prompting the necessity of further research on the anticancer activity (inhibition of cancer cells) of the compound.

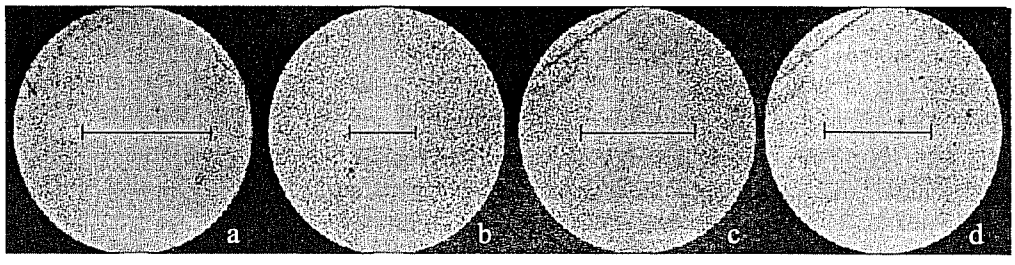

Figure2: Image of the scratch: a- control before incubation b- control, after incubation c- pure compound, before incubation d- Pure compound, after incubation

Structure elucidation of active compound

Low resolution ESI (±) MS analysis gave pseudomolecular peaks at m/z 337 $[M+H]^+$ and 335$[M-H]^-$ revealing molecular mass (MW) to be 336 amu. With the evidence from ^{13}C and ^1H NMR data (see Table 3), it was found that the compound contains 18 C atoms, 22 H atoms and 2-OH groups. The four ^{13}C peaks at 160.19, 182.94, 187.02, 209.16 revealed the presence of four -C=O groups, collectively suggested the molecular formula to be $C_{18}H_{24}O_6$; hence the degree of unsaturation is 7.

Analysis of the ^{1}H-NMR and COSY spectra showed that the multiplet at δ 3.04 correlates with the methyl group at δ 1.23(d) and the multiplet at δ 5.46 correlates with the methyl group at δ 1.34, revealing the spin system CH_3-CH-CH-CH_3. The multiplet at δ_H 1.43 correlates with the -CH_2 group at δ_H 2.39(t) and the –CH_2 group at δ_H 1.57(m) which correlates to the -CH_2 group at δ_H 2.43(t), revealing the spin system $CH_2CH_2CH_2CH_2$.
Structure elucidation of the compound is under way.

Peak No.	δ_c	δ_H (m, J (Hz))	gCOSY	gHMBC
1	12.53	2.07 (3H, s)		15.42, 72.83, 140.23, 144.84, 182.94, 187.02
2	15.42	1.23 (3H, d, 7.2)	3.04	19.02, 40.04, 72.83, 140.23
3	19.02	1.34 (3H, d, 6.0)		15.42, 40.04, 72.83
4	22.60	2.39 (2H, t, 7.8)	1.43	23.58, 27.51, 119.89, 150.75, 187.02
5	23.58	1.57 (2H, m)	2.43	22.60, 27.51, 43.36, 209.16
6	27.51	1.43 (2H, m)	2.39, 1.57	22.60, 43.36, 119.89
7	29.91	2.11 (3H, s)		23.58, 43.36, 209.16
8	40.04	3.04 (1H, m)	1.23, 5.46	72.83, 182.94
9	43.36	2.43 (2H, t, 7.8)		22.60, 27.51, 209.16
10	72.83	5.46 (1H, m)	1.34	15.42, 40.04, 140.23, 160.19
11	119.89			
12	140.23			
13	144.84			
14	150.75			
15	160.19			
16	182.94			
17	187.02			
18	209.16			

Table 3: NMR (600MHz, $CDCl_3$) data for the isolated Compound

CONCLUSION

The compound which is isolated from the fungus *Penicillium citrinum* has a potential anticancer activity.

ACKNOWLEDGEMENT

Financial assistance from NSF research grant RG/2005/HS/11 is gratefully acknowledged.

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