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ANTIMICROBIAL CONSTITUENTS FROM A RHIZOSPHERE *Aspergillus* SPECIES

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INTRODUCTION

As an obvious choice for drug discovery, secondary metabolites of fungi associated with plants and soils of differently stressed environments have been used immensely with profound success. As an initiation of bioactivity guided chemical investigation of fungal metabolites, a fractionation of a bioactive extract from a species of *Aspergillus* was done in order to isolate the responsible constituents.

METHODOLOGY

Isolation, Identification and Growing of Fungal culture

A soil sample associated with roots of *Suaeda maritima* plant collected from the Puttalam District was used to isolate the fungus using standard isolation methods. Purified fungal culture (MW/01/123/01) was identified as an *Aspergillus* sp. by studying the colony characters, fungal morphology and asexual reproductive characters (Onions *et al.* 1981). Species identification is under way using molecular identification methods.

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

Extraction

Broth cultures were filtered under suction, the aqueous filtrate was partitioned with ethyl acetate (EtOAc). Methanol (200mL) was added to the residue (in each 500 mL conical flask) and shaken for 18 hours, filtered under vacuum and the filtrate was concentrated at 40 °C under vacuum to $1/5^{th}$ of the original volume to remove methanol. The remaining extract was partitioned with EtOAc. The EtOAc extracts were combined and evaporated to dryness under vacuum at 40 °C to yield the fungal extract.

Methanol (100 mL) was added to each solid culture bottle and was shaken for 24 hrs on a rotary shaker and filtered. Filtrate was concentrated at 40 °C under vacuum to $1/5^{th}$ of the original volume to remove methanol. The remainder was partitioned with EtOAc.

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 out in which fraction the active constituents are present.

The CH_2Cl_2 fraction, which showed antibacterial activity was subjected to chromatography over a column of Sephadex LH20 (40g) made up in CH_2Cl_2: hexane 4:1 and eluted with CH_2Cl_2: hexane 4:1, CH_2Cl_2: acetone 3:2, CH_2Cl_2: acetone 1:4, CH_2Cl_2: MeOH 1:1, and MeOH. Column fractions were pooled according to their TLC patterns. Fractions, which showed significant antibacterial

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activity, were subjected to further fractionation using silica column chromatography followed by preparative TLC separations to isolate the bioactive constituents.

Antibacterial Assay

The extracts and/or fractions dissolved in MeOH were used 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). Filter paper discs containing test samples (extracts dissolved in MeOH) were made and dried for 24 hours to remove the solvent prior to the experiment. The dose of test samples was 500 µg / disc at initial steps of fractionation and at later stages (after Sephadex column) it was reduced according to the weight percentage of the fractions. A uniform lawn of each bacterial species was prepared. Discs were placed on each plate. All experiments were done in duplicate and were incubated at ~ 35 °C for ~ 24 hours. The zone of inhibition if present was measured along two axes, perpendicular to each other and average value was calculated. As the zone of inhibition depends on the strength of the bacterial lawn, the bacterial inoculum was standardized by maintaining a pre-determined optimum density value. A disc containing 25 µg of Amoxicillin was used as a positive control to compare the activities of the test samples while a disc soaked with 10µL of MeOH and dried was used as a negative control.

RESULTS AND DISCUSSION

Fractionation of the Ethyl acetate Extract and Isolation of Antibacterial Constituents

The crude EtOAc extract obtained from the small scale Potato Dextrose Broth (PDB) culture of MW/01/123/01, when subjected to antibacterial assay showed antibacterial activity against *Bacillus sp.* and *Staphylococcus sp.* giving average clear zones of 18.3 mm and 18.5 mm, while amoxicillin (positive control) gave 12.5 mm and 31.75 mm respectively. Prompted with these positive results, an experiment was set up to find out the best growth medium to grow the fungus at a large scale for the isolation and characterization of antibacterial metabolite.

The fungal culture was inoculated into Yeast Malt Agar (YMA) (3 x 40 mL), Potato Dextrose Agar (PDA) (3 x 40 mL), Yeast Malt Broth (YMB) (250 mL) and PDB (250 mL), and incubated at 30 °C for 21 days. Broth cultures (YMB and PDB) and solid cultures (YMA and PDA) were extracted as described in the methodology to obtain EtOAc extracts. The antibacterial activity was determined for all four EtOAc extracts.

Table 1 summarizes the yields and results of the antibacterial test of EtOAc extracts obtained from different culture media. Although the both broth cultures gave higher yields when extracted with EtOAc, it was clearly evident that PDA to be the best medium for the growth of this particular fungus in large scale as this extract showed the highest antibacterial activity.

Culture Medium	Volume of medium (mL)	Weight of the Extract (mg)	Dose (µg/disc)	Diameter of Clear Zone (mm)
YMA	40 x 3	28.7	500	12.5
PDA	40 x 3	39.3	500	20.5
YMB	250	89.7	500	17.5
PDB	250	55.9	500	13.0
Positive control: Amoxicillin			25	18.0

Table 1: Effect of the different culture media on the yield and the antibacterial activity of EtOAc extracts on *Bacillus* sp.

The fungal culture was inoculated into 100 bottles (350mL) each containing 40 mL of PDA and incubated at 30 °C for 21 days and the secondary metabolites were extracted into EtOAc as previously described. The extract thus obtained, showed antibacterial activity (Diameter of clear zone: 17 mm) against *Bacillus* sp. The EtOAc extract (2.03 g) was subjected to solvent partitioning and the resulted fractions were subjected to bioassay at 500µg per disc (See Table 2).

Fraction	Weight (g)	Dose (µg/disc)	Diameter of Clear Zone (mm)
Hexane	0.383	500	13.5
CH_2Cl_2	1.228	500	20.0
50% aqueous MeOH	0.391	500	14.5
Positive control: Amoxicillin		25	10

Table 2: Fractions obtained from solvent partitioning and activity against *Bacillus* sp.

As the 50% aq.MeOH fraction also showed antibacterial activity the remaining aqueous MeOH layer was re-extracted with CH_2Cl_2 followed by EtOAc and subjected to antibacterial assay, which confirmed that the 50% aq. MeOH fraction and the EtOAc fraction are devoid of any activity. After the confirmation of the bioactivity, the two CH_2Cl_2 fractions were combined (1.402g).

The CH_2Cl_2 fraction (1.329 g) was subjected to chromatography over a column of sephadex LH20 as described in the methodology section. Column fractions were pooled according to their TLC patterns and 17 major fractions were obtained and were tested for their antibacterial activity against *Bacillus* sp. Table 3 summarizes the details of the column fractions, which showed the antibacterial activity.

Solvent	Fraction No	Weight(mg)	Diameter of Clear Zone (mm)
CH_2Cl_2/hexane 4:1	f2	153.5	26
	f3	69.8	13
	f4	55.6	30
	f5	7.0	10
CH_2Cl_2/acetone 3:2	f6	21.6	9.5
	f7	116.0	10.5
	f8	140.1	8
	f9	350.5	12.5
	f10	128.9	17
	f11	32.4	9.5
	f12	31.4	8.5
	f13	27.2	9.5

Table 3: Sephadex LH-20 Column fractions of the CH_2Cl_2 fraction and antibacterial activity against *Bacillus* sp.

Fractions 1 and 14 to 17 did not show any activity. As the fraction F-10 was considerably pure and had a significant antibacterial activity, it was subjected to bioactivity guided fractionation using silica column chromatography followed by preparative TLC separations to isolate the bioactive compound C1 (a yellow crystalline compound). From TLC studies it was found that fraction f2 (which showed antibacterial activity more than the fraction f10) is not composed of C1, which prompted fractionation of f2 for isolation of a second bioactive compound C2 (a white crystalline compound).

By subjecting the compounds for several bioassays, it was found that 150 µg of C1 showed the same activity of 25 µg of Amoxicillin while 25 µg of C2 showed a higher activity than the same dose of Amoxicillin could (See Table 4).

Test Sample	Dose (µg/disc)	Diameter of Clear Zone (mm)
Compound 1 (C1)	150	12.5
Compound 2 (C2)	25	18.0
Positive control: Amoxicillin	25	12.5

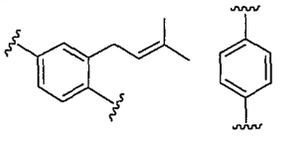
Table 4: Antibacterial activity of pure compounds against *Bacillus* sp.

NMR data were obtained for the compound 1 (C1) and are summarized in Table 5.

Peak No.	δ_c	δ_H (m, J (Hz))	gHMBC
1	17.61	1.57 (3H, s)	25.63, 121.82, 133.30
2	25.63	1.63 (3H, s)	17.61, 121.82, 133.30
3	28.43	3.08 (2H, d, 7.2)	121.82, 126.91, 133.30, 153.39
4	38.58	3.39(2H, m)	85.77, 124.20, 128.26, 131.66, 170.21
5	53.40	3.72 (3H, s)	170.21
6	85.77		
7	114.52	6.47 (1H, d, 8.0)	124.20, 126.91, 153.39
8,9	115.78	6.84 (2H, dd, 2.0, 8.8)	121.90, 157.46
10	121.82	5.05 (1H, t, 1.6)	17.61, 25.63
11	121.90		
12	124.20		
13	126.91		
14	128.26		
15	128.95	6.54 (1H, dd, 2.0, 8.0)	38.58, 124.20, 126.91, 131.66, 153.39
16,17	129.33	7.54 (2H, dd, 2.0, 8.8)	128.26, 157.46
18	131.66	7.54 (1H, d, 2.0)	28.43, 38.58, 128.95, 153.39
19	133.30		
20	137.44		
21	153.39		
22	157.46		
23	169.44		
24	170.21		

Table 5: NMR (400MHz, $CDCl_3$) data for Compound 1(C1)

Structure of the compound 1(C1) was partially elucidated by analyzing 1D and 2D NMR data and following fragments were identified.



Possible structure fragments of compound C1

Structure elucidation of both compounds C1 and C2 is under way.

CONCLUSION

The *Aspergillus* sp. selected for the present study has produced secondary metabolites with significant antibacterial activity against gram positive bacteria including *Bacillus* sp.

ACKNOWLEGEMENT

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