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Studies on Antimicrobial Activities of *Chaetomium atrobrunneum* Ames against Selected Microorganisms

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Abstract

The antibacterial and antifungal activities of *Chaetomium atrobrunneum* was investigated against *Staphylococcus aureus* (Gram +ve), *Escherichia coli* (Gram –ve) and *Candida albicans* using well diffusion and dilution method. The solvents used for extraction experiment was Ethylacetate and Dichloromethane and were removed in vacuo to yield viscous oils and paste which were made upto M_1D DCM extract-0.04g, M_1D EtOAc extract-0.03g, PDB EtOAc extract-0.04g, YPS EtOAc extract-0.025g dissolved in 10ml of DMSO each respectively. These were tested in varying volumes of 100-200µl/plate. Ampicillin and Itraconazole were used as references for bacteria and fungi . The solvent extracts of *Chaetomium atrobrunneum* showed higher antimicrobial inhibitory activity at 0.8mg/200µl of PDB EtOAc extract plate and the antioxidant activity test has been done with this PDB EtOAc extract. The extract which showed the higher antimicrobial activity is PDB-ethylacetate extract was taken for antioxidant study.

Key Words: *Chaetomium atrobrunneum*, Antimicrobial activity, *Escherichia coli*, *Staphylococcus aureus*, *Candida albicans*, Antioxidant activity

Introduction

An antimicrobial is a compound that kills or inhibits the growth of microbes such as bacteria (antimicrobial activity), fungi (antifungal activity), viruses (antiviral activity) or parasites (antiparasitic activity). This study discusses the antimicrobiological (antibacterial and antifungal) activity of an endophyte *Chaetomium atrobrunneum*. Its antimicrobial properties were studied against *Staphylococcus aureus, Escherichia coli* and *Candida albicans* using well diffusion, and dilution method.

Microbes that colonise living, internal tissues of plants without causing any immediate negative effects [2], synergistic to their host and atleast some of them are thought to be useful to the plant by producing special substances, such as secondary metabolites, that prevent the host from being attacked successfully by fungi and pests [29].

Chaetomium is an ascomycetous filamentous fungus can be isolated from soil, air and also from composing plant debris, especially woody or straw-like materials. Some species are thermophilic and neutrophilic in nature. The genus *Chaetomium* contains a number of species such as *C.atrobrunneum, C. globosum, C.funicola* and *C.strumarium*. It occur widely in nature and certain species ability to produce biologically active metabolites such as Chaetoglobosin, Isochochlinidol etc and may produce a plethora of substances of potential use to modern medicine, agriculture and pharmaceutical industry.

The pathogenic microbes such as *Escherichia coli, Staphylococcus aureus and Candida albicans* were used. *Escherichia coli* can cause several intestinal and extra intestinal infections such as urinary tract infections, meningitis, peritonitis, mastitis, septicemia and gram-negative pneumonia. *Staphylococcus aureus* can cause furuncles (boils), carbuncles (a collection of furuncles). In infants, *Staphylococcus aureus* can cause a severe disease Staphylococcal scalded skin syndrome (SSSS). Staphylococcal *endocarditis* (infection of the heart valves) and pneumonia may be fatal. *Candida albicans* is a diploid fungus (a form of yeast) and is a casual agent of opportunistic oral and genital infections in humans.

Antioxidant activity was evaluated for the extract which shows the higher inhibition in antimicrobial activity tests.

The purpose of the present study was to extract, explore and characterize antimicrobial activity produced by the endophytic fungus *Chaetomium atrobrunneum* isolated from *Michaelia champaca.L*.

Materials and Methods

Collection of plant materials

The leaf parts of *Michaelia champaca.L*. was collected from Kodaikanal, India (Tamil Nadu). The leaves are washed with running tap water to remove the dust from the surface. By following the modified method of surface sterilization [3] the leaves are cut into small pieces of 0.5cm diameter. Then it were surface sterilized by immersion in 70% Ethanol for 5s, followed by 4% Sodium hypochlorite for 20s and finally rinsed in sterile distilled water for 10s. The excess moisture was blotted on a sterile filter paper. The surface sterilized segments were evenly spaced in petriplate containing potato dextrose agar medium. Then the plates were sealed using parafilm and incubated at 26±1°C in a 12 hrs of light/dark

cycles and monitored everyday for the growth of endophytic fungal colonies from the segments. Then the hyphal tips of well grown fungi was isolated and brought into pure culture. Then identification of the fungus depends on morphological and microscopical examination.

Production and extraction

The isolated endophytic fungus *Chaetomium atrobrunneum* was inoculated into different production medium include M_1D medium, PDB medium, YPS medium in Erlenmeyer flask and incubate it for 2 to 3 weeks. It was extracted using with different solvents like Dichloromethane and Ethylacetate. These solvents were removed in vacuo using a rotor vapourator. The extracts were placed in vials.

Antimicrobial activity tests

1. Making up extract solution- 0.04g of M_1D DCM extract, 0.03g of M_1D EtOAc extract, 0.04g of PDB EtOAc extract and 0.025g of YPS EtOAc extract was weighed and add the 10ml of DMSO solvent to make it.
2. Microorganisms- *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 25922) *and Candida albicans* (MTCC 227) were used for the present study.
3. Agar preparation- Two types of agar were used, nutrient agar to make up the medium for bacteria and PDA (Potato Dextrose Agar) to make up the medium for fungi.
4. Potato Dextrose Agar- The potato was peeled and 100g was measured, finely chopped and boiled to a mash in a distilled water. The dextrose and agar was measured (12.5g) each respectively and add to the flask. Then it was made upto 500ml using distilled water. The flask was autoclaved at 121°C for 20 mins and pH range maintained between 6.5-7.0. Under aseptic conditions, the medium has to poured into sterile plates and allow it to solidify.
5. Nutrient agar- Peptone(2.5g), sodium chloride(2.5g), beef extract(1.5g) and agar(10g) was measured and added in 1L erlenmeyer flask. It was suspended in 500ml of distilled water, stirred, boiled to dissolve and then autoclaved at 121°C for 20 mins. The pH range between 7.0±0.2. Under aseptic conditions the medium was poured into sterile plates.
6. References and control- The references were antibiotic in nature. Ampicillin and Itraconazole. Ampicillin used as reference for bacterial species used *Escherichia coli, Staphylococcus aureus*. Itraconazole was used as reference for the fungus *Candida albicans.* The pure solvent (DMSO) can be used as a control.
7. Well diffusion method- Under aseptic conditions, the autoclaved nutrient agar and PDA medium was poured into sterile plates and allow it to solidify. After solidification, one day bacterial culture was applied onto the plates of NA medium and fungal culture was applied onto the PDA medium plates. Then, the well was made by using 6mm cork borer that was sterilized with alcohol and flame. The *C.atrobrunneum* extract dissolved in concentration of M_1D DCM extract-0.04g, M_1D EtOAc extract-0.03g, PDB EtOAc extract-0.04g and YPS EtOAc extract-0.025g in 10ml of DMSO was pipetted into the different wells in a separate plates for bacteria and fungi respectively, using a microliter syringe. The reference ampicillin (bacteria)-0.03g dissolved in 10ml of DMSO and itraconazole (fungi)-0.03g dissolved in 0.1N of HCL in 1ml of water. The plates were labelled, covered and incubated for 24hrs for bacteria and 48hrs for fungi respectively.
8. Dilution method- For this method, the LB (Luria-Bertani) broth was prepared. It is a rich medium to culture bacteria such as *Eschericia coli, Staphylococcus aureus*. Tryptone(2.5g), sodium chloride (2.5g), yeast extract (1.5g) were measured and added to make up 500ml in 1L Erlenmeyer flask and pH of broth was adjusted to 7.4 using sodium hydroxide. Then 5ml of broth was resuspended in all test tubes and autoclaved at 121°C for 20 mins. This method is used to test the extracts of *C. atrobrunneum* for antimicrobial activities against bacteria by investigating whether there was turbidity or not. Turbidity represents growth of microbes, while no turbidity represents inhibition of microbes. One set of test tubes containing LB broth was inoculated with *S.aureus* and other set inoculated with *E. coli* using a loop, flame and alcohol. The *C. atrobrunneum* solvent extracts M_1D DCM extract-0.04g, M_1D EtOAc extract-0.03g, PDB EtOAc extract-0.04g and YPS EtOAc extract-0.025g in 10ml of DMSO each respectively. Then the inoculated tubes were treated with solvent extracts and then the tubes were observed after 24hrs.

Antioxidant Activity test

Superoxide anion scavenging activity

The method of *Markulund* (1974) [20] modified by *Ekanayake et al*., (2004) [4] was used in this test. The method is based on the inhibition of the autoxidation of pyrogallol by phenolic compounds. To the assay mixture composed of a phosphate buffer solution (2.6 ml, 50 mM in water, pH 8.22 ± 0.03) with the analytical sample extract (0.3 ml) was added a freshly prepared solution of pyrogallol (0.1 ml of a 3 mM solution of pyrogallol in 0.010 M HCl). The autoxidation reaction rate of pyrogallol was determined at 325 nm by monitoring the absorbance every 30s for a total period of 10 min, corresponding to the end of the reaction. The scavenging activity of the superoxide anion (O^{-2}) was calculated by the following formula [27]:

$$\% \text{ of Scavenging activity} = \frac{(K_0 - K_1)}{K_0} \times 100$$

Where K0 and K1 are autoxidation rates of the pyrogallol without and with the fungal extract, respectively.

Thin Layer Chromatography (TLC)

TLC analysis was carried out on 0.25mm silica gel precoated plates. The plates were developed by the solvent system. The compound was detected with 1% vanillin sulfuric acid (w/v) and heating. It appears as a bluish spot that faded to dark grey after 24 hours. Then the area of the plate containing putative compound was carefully removed by scraping off the silica at the appropriate Rf.

Results

Antimicrobial activity test

These extracts obtained from *Chaetomium atrobrunneum* were made in the concentration of M_1D-DCM extract (0.04g/10ml of DMSO), M_1D-EtOAc extract (0.03g/10ml of DMSO), PDB-EtOAc extract (0.04g/10ml of DMSO), YPS-EtOAc extract (0.025g/10ml of DMSO). PDB-EtOAc extract of *C. atrobrunneum* showed maximum zone of inhibition by comparing with the reference antibiotic Ampicillin (0.03g/10ml of DMSO).

Table:1 Results of Well diffusion method for Sample Extract against bacteria

Extract	Volume of Extract (µl)	Diameter of Zone of inhibition of *E. coli* (mm^2)	Diameter of Zone of inhibition of *S. aureus* (mm^2)
M_1D extract with Dichloro - methane (0.04g/10ml DMSO)	100	23	12
	150	24	12
	200	25	13
M_1D extract with Ethyl Acetate (0.03g/10ml DMSO)	100	22	11
	150	22	12
	200	23	13
PDB extract with Ethyl Acetate (0.04g/10ml DMSO)	100	23	11
	150	24	14
	200	26	15
YPS extract with Ethyl Acetate (0.025g/ml DMSO)	100	19	11
	150	21	13
	200	23	14
Reference (Ampicillin) (0.03g/10ml DMSO)	100	25	16
	150	26	18
	200	28	19

PDB-EtOAc extract (0.04g/10ml of DMSO) of *Chaetomium atrobrunneum* showed maximum zone of inhibition by comparing with the reference antibiotic Ampicillin (0.03g/10ml of DMSO)

Table:2 Results of Well diffusion method for Sample Extract against *Candida albicans*

Extract	Volume of Extract (µl)	Diameter of Zone of inhibition of *Candida albicans* (mm^2)
M_1D extract with Dichloro-methane (0.04g/10ml DMSO)	100	19
	150	21
	200	22
M_1D extract with Ethyl Acetate (0.03g/10ml DMSO)	100	18
	150	21
	200	22
PDB extract with Ethyl Acetate (0.04g/10ml DMSO)	100	21
	150	23
	200	24
YPS extract with Ethyl Acetate (0.025g/ml DMSO)	100	13
	150	15
	200	16
Reference (Itraconazole) (0.03g/0.1N of HCL in 1ml of water)	100	14
	150	15
	200	17

PDB-EtOAc extract (0.04g/10ml DMSO) of *Chaetomium atrobrunneum* showed maximum zone of inhibition by comparing with the reference antibiotic Itraconazole (0.03g/0.1N of HCL in 1ml of water)

Dilution method

T0 = No Turbidity = Inhibition
T1 = Lightly Turbid = Moderately Inhibited
T2 = Moderately Turbid = Lightly Inhibited
T3 = Very Turbid = No Inhibition

Table: 3 Shows degree of turbidity of dissolved *C.atrobrunneum* extract in different solvents at different volumes against *Escherichia coli*

C.atrobrunneum extract dissolved in solvents at concentration of	Volume of dissolved extract 100 µl	Volume of dissolved extract 150µl	Volume of dissolved extract 200 µl
M_1D extract with Dichloro –methane (0.04g/10ml DMSO)	T_2	T_1	T_0
M_1D extract with Ethyl Acetate (0.03g/10ml DMSO)	T_2	T_1	T_0
PDB extract with Ethyl Acetate (0.04g/10ml DMSO)	T_2	T_1	T_0
YPS extract with Ethyl Acetate (0.025g/ml DSMO)	T_3	T_2	T_1
Reference (Ampicillin) (0.03g/10ml DMSO)	T_0	T_0	T_0

Table:4 Shows degree of turbidity of dissolved *C. atrobrunneum* extract in different solvents at different volumes against *Staphylococcus aureus*

C. atrobrunneum species extract dissolved in solvents at concentration of	Volume of dissolved extract 100 µl	Volume of dissolved extract 150 µl	Volume of dissolved extract 200 µl
M_1D extract with Dichloro –methane (0.04g/10ml DSMO)	T_3	T_3	T_2
M_1D extract with Ethyl Acetate (0.03g/10ml DMSO)	T_3	T_2	T_2
PDB extract with Ethyl Acetate (0.04g/10ml DMSO)	T_2	T_1	T_0
YPS extract with Ethyl Acetate (0.025g/ml DMSO)	T_3	T_3	T_3
Reference (Ampicillin) (0.03g/10ml DMSO)	T_1	T_0	T_0

The zone of inhibition of bacteria includes *E.coli* and *S.aureus* is higher in PDB-EtOAH extract when compared with the other extracts used was M_1D-DCM, M_1D-EtOAc, YPS-EtOAc. The zone of inhibition act against the bacteria was in the order of following sequence PDB-EtOAc extract > M_1D-DCM extract > M_1D-EtOAc extract > YPS-EtOAc extract.

Graphs

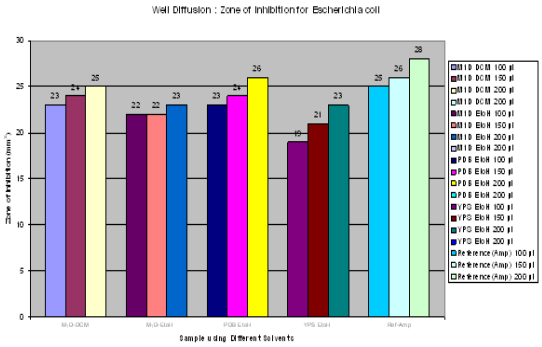

Fig.1 Well Diffusion: Zone of Inhibiton of *Escherichia coli*

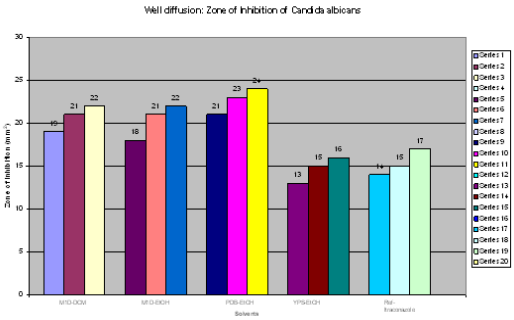

Fig.3 Well Diffusion: Zone of Inhibiton of *Candida albicans*

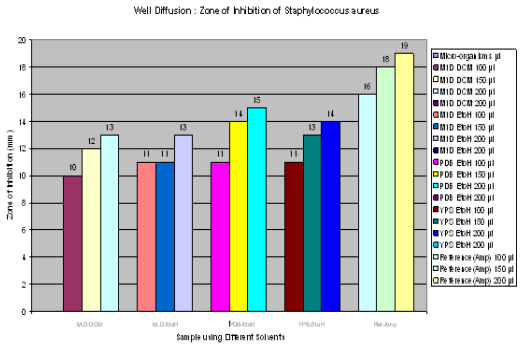

Fig.2 Well Diffusion: Zone of Inhibiton of *Staphylococcus aureus*

Antioxidant activity

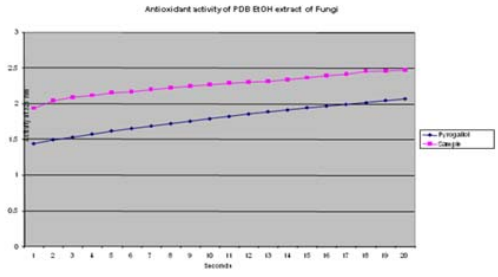

Fig.4 Antioxidant activity of PDB-EtOAc extract of Fungi

Dioxygen form superoxide anions O_2^- by a single electron transfer during the pyrogallol autoxidation in basic solutions. The superoxide anions are scavenged by antioxidants and consequently, decrease the rate of pyrogallol autoxidation or even inhibit it.

Discussion

Endophytes or any type of fungus are capable of producing novel secondary metabolites as the reports says many of the endophytes are still unknown and the compound are produced by the respective fungus are still remain unknown. So with this view, the *Chaetomium atrobrunneum* is taken for testing its production for secondary metabolites. Antimicrobial activities of compounds biosynthesised by the plant endophytes have been reported only by few researchers [5, 6, 12, 23, 25, 26]. The bioassay method is very useful for applying to the screening for antimitotic and antifungal activities of secondary metabolites fron various natural sources and it is quick and easy method [11]. Each of approximate extracts includes M_1D-DCM extract, M_1D-EtOAc extract, PDB-EtOAc extract, YPS-EtOAc extract obtained was mixed with 10ml of DMSO taken for antimicrobial activity by well diffusion method were successful in determining *C. atrobrunneum* producing metabolite having antimicrobial activity. The maximum zone of activity against *E.coli* and *S.aureus* in PDB-Ethylacetate extract shows 26mm^2 and 15mm^2 of 200µl concentration when compared with other extracts such as M_1D-DCM extract > M_1D-EtOAc extract > YPS-EtOAc extract and it was compared with reference antibiotic (Ampicillin) for antibacterial activity.

The well diffusion method was used against *C.albicans* indicates that maximum zone of inhibitory activity of 24 mm^2 in PDB-EtOAc extract of 200µl concentration when compared with other extracts such as M_1D-DCM extract > M_1D-EtOAc extract > YPS-EtOAc extract was compared with reference antibiotic (Itraconazole) for antifungal activity.

The dilution method was used to test the fungal extracts for antimicrobial activity against bacteria such as *E.coli* and *S.aureus*. The fungal extract which showed the positive result for the well diffusion method were used and checked for the turbidity method. LB broth as an enrichment medium for bacteria (*E.coli* and *S.aureus*) were used for this method and noted for results. T_0 - Nil growth were seen in PDB-EtOAc extract, M_1D-DCM extract, M_1D-EtOAc extract at 200µl concentration were lesser turbidity in 100 and 150µl concentration. The reference antibiotic ampicillin and control showed inhibitory and non-inhibitory activity as predicted earlier.

The extract which showed the higher antimicrobial activity is PDB-ethylacetate extract was taken for antioxidant activity. The superoxide anions are scavenged by the antioxidants and consequently, decrease the rate of pyrogallol, autoxidation or even inhibit it. The ability of the phenolic compounds from extract to scavenge the superoxide anion was carried out using the pyrogallol autoxidation method [22]. The results are reported in fig.4 were the PDB-ethylacetate extract of *Chaetomium atrobrunneum* was effective superoxide anions scavenger at a concentration of 300µl/37.13% value of higher scavenging activity.

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